A study of the

HUMORAL AND CELLULAR IMMUNE RESPONSE

to

Saccharomyces cerevisiae

in man

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MATERIALS AND METHODS

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Declaration

This thesis was composed entirely by me, the undersigned.

All of the work described herein is my own, except where otherwise indicated in the text.

C. J. DARROCH

List of abbreviations

The following abbreviations have been used. Most have also been defined in the text.

AAG	α_1-acid glycoprotein (orosomucoid)
AET	2-aminoethylisothiouronium bromide hydrobromide
APC	antigen-presenting cell(s)
αLA	α-lactalbumin
BSA	bovine serum albumin
βLG	β-lactoglobulin
CBMC	cord blood mononuclear cell(s)
CI	confidence interval
cpm	counts per minute
CTAB	cetyltrimethylammonium bromide (cetavlon)
DEAE	diethylaminoethyl-
ELISA	enzyme-linked immunosorbant assay
FS	forward scatter
HPLC	high-performance liquid chromatography
hsp	heat-shock protein
IDDM	insulin-dependent diabetes mellitus
IEL	intra-epithelial lymphocyte
IFN	interferon
Ig	immunoglobulin
IL	interleukin
LAK	lymphokine-activated killer
LDA	limiting dilution analysis
LPL	lamina propria lymphocyte
NK	natural killer
PAS	periodic acid Schiff's reagent
PBMC	peripheral blood mononuclear cell(s)
PBS	phosphate-buffered saline
PCFIA	particle-concentration fluoroimmunoassay
PHA	phytohaemagglutinin
PPD	purified protein derivative of *M. tuberculosis* (tuberculin)
PWM	pokeweed mitogen
SDS-PAGE	sodium dodecyl-sulphate polyacrylamide gel electrophoresis
SEC/MALLS	size-exclusion chromatography/multi-angle laser light scattering
SI	stimulation index
SK or SK/SD	streptokinase/streptodornase
SRBC	sheep red blood cell(s)
SS	side scatter
rpm	revolutions per minute

ABSTRACT

Saccharomyces cerevisiae (bakers'/ brewers' yeast) is a ubiquitous dietary constituent in the developed world. Previous studies, using semi-quantitative ELISA techniques, suggested that patients with Crohn's disease have higher titres of IgG and IgA isotype-specific antibodies to this yeast than are found in normal control subjects or patients with ulcerative colitis. For this study, in order to allow more stringent assay standardisation and more meaningful numerical comparison of the relative antigen-binding capacities of different sera, a quantitative ELISA was developed for measurement of anti-yeast antibodies, using a soluble extract of yeast (sacc) as the antigen. The finding of raised levels of yeast antibodies in Crohn's disease was confirmed, and the data suggest that this may be related to the presence of disease in the small bowel, although this latter observation did not reach statistical significance. Patients with chronic liver disease also had higher antibody levels than controls, but less markedly so than in Crohn's disease. When sera were tested in a similar assay for antibodies to bovine casein, no difference was found between controls and the Crohn's or liver disease group.

The response of peripheral blood mononuclear cells (PBMC) to sacc was examined using a proliferation assay measuring uptake of tritiated thymidine. Cells from normal controls demonstrated dose-dependent proliferation, the time-course of which resembled that obtained with known recall antigens. Following separation of cell populations by rosetting with sheep erythrocytes, the responding cells were shown to be T-lymphocytes and the magnitude of the response was sensitive to the number of antigen-presenting cells present in the culture. When positive selection with immunomagnetic beads was used to further separate T-cells into highly purified $CD4^+$ and $CD8^+$ populations, responsiveness to yeast co-separated with the $CD4^+$ subset. Following negative selection of cells expressing CD45RO or CD45RA, responsiveness was largely, but not exclusively, confined to the $CD45RO^+$ population. Limiting dilution analysis of peripheral blood T-cells gave estimates of the sacc-specific precursor cell frequency in keeping with values previously reported for recall antigens, although the experimental data could not be shown to conform to single-hit kinetics. By sequential stimulation in long term culture, it was possible to obtain populations of cells which were uniquely responsive to sacc but unresponsive to other recall antigens. At some concentrations of sacc, proliferation responses of PBMC from Crohn's disease patients were higher than those in normal subjects, but the difference was not convincing overall.

Digestion of sacc with pronase abolished the T-cell response but left specific antibody-binding intact, supporting the suggestion that antibody recognition is dependent on carbohydrate epitopes. Yeast cell wall mannan is implicated as the likely site of B-cell epitopes; evidence pertaining to T-cell epitopes is less conclusive.

Thus, this study provides evidence that immune sensitisation to a common dietary constituent frequently occurs in the normal population, leading to detectable humoral and cellular immune responses. The T-cell response appears to be genuinely antigen-specific, and not due to non-specific lymphocyte activation. The gastrointestinal lymphoid system may be the site at which primary sensitisation occurs. In patients with Crohn's disease, the humoral response is enhanced, possibly as a consequence of inflammatory processes in the small bowel.

INTRODUCTION

Background: *S. cerevisiae* and Crohn's disease

In 1988, Main *et al.*,[1] using a boiled suspension of *Saccharomyces cerevisiae* (bakers'/brewers' yeast) as antigenic substrate in an ELISA, reported specific antibodies of IgG and IgA isotype in patients with Crohn's disease, which distinguished them from both normal subjects and those with ulcerative colitis. Subsequently, it was shown that this observation held true for specific IgG against 11 out of 12 different strains of this yeast, whereas there was no difference between groups for antibodies to *Candida albicans*.[2] However, there was considerable variation between patients with respect to their relative antibody response to different *S. cerevisiae* strains, and strain-specific antigenic heterogeneity was subsequently demonstrated by ELISA inhibition studies.[3] Interestingly, the strain against which Crohn's disease patients had *not* demonstrated increased antibodies appeared to be antigenically more related to *C. albicans* than other *S. cerevisiae* strains.

Barnes *et al.*[4] also found IgG anti-*S. cerevisiae* in a larger proportion (63%) of Crohn's disease patients than in normal controls (8%), ulcerative colitis (15%), coeliac disease (15%), dermatitis herpetiformis (10%), irritable bowel syndrome (20%) and atopic eczema (16%). Furthermore, IgA anti-*S. cerevisiae* was present in 43% of the Crohn's disease group but in only 1 of 60 normal controls and in none of the other disease groups. In contrast, the prevalence of IgG antibodies to *E. coli* was increased equally in Crohn's disease and ulcerative colitis, whereas IgG antibodies to cow's milk were detected with increased frequency in ulcerative colitis but *not* Crohn's disease, and IgG antibodies to gliadin and ovalbumin were no more

frequent than normal in either of the inflammatory bowel diseases.

In a study of twins who were concordant or discordant for Crohn's disease or ulcerative colitis, Lindberg *et al.*[5] measured IgG, IgA and IgM isotype-specific antibodies to extract of whole *S. cerevisiae* as well as a commercial preparation of mannan from *S. cerevisiae*, ovalbumin, bovine β-lactoglobulin and gliadin. Compared with matched controls, twins with Crohn's disease had significantly elevated antibodies of all three isotypes against mannan, and of IgA against whole yeast extract, in the presence of normal or decreased antibodies to the other dietary antigens; IgG to yeast extract was higher in those with disease exclusively affecting the small bowel than in those with large bowel disease (with or without small bowel disease). Healthy twins of those with Crohn's disease also had elevated IgA against mannan and yeast extract, but this was less marked than in those with disease. Among twins with ulcerative colitis, there was a modest elevation of IgA against mannan and gliadin, but this was slightly less than in their healthy twins. Only two of the subjects in this study (both of whom had Crohn's disease) were said to have had active disease.

Giaffer *et al.*[6] compared IgG and IgA antibodies to three *S. cerevisiae* strains in Crohn's disease, ulcerative colitis and coeliac disease. Specific IgG to all three strains was raised in both Crohn's disease and coeliac disease, whereas specific IgA to all three strains was raised in Crohn's disease only. Crohn's disease subjects with small bowel disease (with or without large bowel disease)[a] had higher levels of IgG to two yeast strains, and IgA to one strain, than those with exclusively large bowel

a: there is some internal contradiction in this paper regarding whether patients were, in fact, classified in this way

disease.

These studies continued an established theme of investigating the possibility of abnormal immune responsiveness to dietary antigens in gastrointestinal disease. However, in addition, they introduced *S. cerevisiae* as a novel potential antigen — so far as can be ascertained, the existence and nature of the normal human immune response to this organism has not previously been investigated.

This study, therefore, represents an attempt not only to expand on previous work on bakers' yeast in the context of inflammatory bowel disease, but also to describe the immune response in normal subjects, with particular reference to cellular immunity and antigen-specificity, and to formulate and test hypotheses concerning the nature of the antigenic substrate(s) involved.

In the remainder of this section, material will be introduced which is of wider historical or technical relevance to the data which are then presented. The latter will then be discussed in the context of this, and other, information.

Foods as antigens

CIRCULATING ANTIBODIES TO DIETARY ANTIGENS

Historically, the impetus for investigating the humoral response to dietary antigens has been two-fold: firstly, there has been a need to arrive at a phenomenological description of normal immunity from which basic mechanisms might be deduced; secondly, there has been considerable interest in examining how such responses may be modified by different disease states, thus providing insight into aetiopathology, or information useful in diagnosis or management of patients.

Naturally, much of the research in this area has been ultimately concerned

with pathological conditions which are overtly associated with adverse clinical reactions to dietary components, food allergy *per se* being the most obvious example. In this context, the biological significance of antigen-specific IgE as a cause of type 1 (immediate, anaphylactic) hypersensitivity reactions is well-established, and will not be discussed further. However, in the course of these investigations, many observations have been made on the development of antibody responses in normal subjects and in those with a variety of pathologies.

Observations in normal subjects

> *"Circulating antibodies to ingested proteins can be detected so commonly that their presence must be considered a natural occurrence."*[7]

Because of its nutritional importance, well-characterised protein composition and frequent association with gastrointestinal intolerance, the overwhelming majority of studies of antibodies to dietary antigens have been concerned with those specific for cow's milk antigens.

In 1965, Rothberg and Farr,[8] using a radioimmunoassay developed by the latter, carried out a quantitative examination of circulating antibodies to bovine serum albumin (BSA) and α-lactalbumin (αLA), in a large sample of nine hundred children and adults, comprising both normal subjects and those suffering from a range of unrelated conditions. The most striking finding related to the way in which the prevalence of antibodies of either specificity depended on the age of the subjects at the time of testing: up to 3 months of age, anti-BSA was present in about 1/3 of infants, the same prevalence as in adults of reproductive age (16–40 years), and therefore in keeping with transplacental, passive acquisition; thereafter, prevalence of

anti-BSA increased to 61% between the ages of 6 and 15 years, and then declined to <10% over the age of 40 years. Furthermore, when the antigen-binding capacity of positive sera was quantified, all of those with a high binding capacity were from children. Compared with healthy subjects, prevalence of anti-BSA was increased among children with iron deficiency anaemia (who were also drinking large amounts of milk) and among adults with allergic diseases, including asthma, presumed to be unrelated to milk. Although the *prevalence* of anti-BSA was not increased among adults with gastrointestinal disease, positive sera from this group had the highest *antigen-binding capacity* of any of the adult groups. Antibodies to αLA showed a similar age distribution to, but were about half as prevalent as, anti-BSA.

Subsequently, Korenblat *et al.*[9] investigated possible reasons for the age-dependent decline in anti-BSA. Neither re-establishing childhood patterns of oral intake of BSA, nor parenteral immunisation with the antigen, could induce an antibody response in adult subjects who had become seronegative, although titres increased among those who had continued to produce antibody. Therefore, it was inferred that non-production of anti-BSA by adults was due to a state of acquired immunological unresponsiveness rather than relative lack of exposure to, or altered gut handling of, antigen.

These findings have been confirmed and extended by other studies, some of which have also examined the effect of delayed antigen exposure on the development of antibody responses. For example, Kletter *et al.*,[7,10] using radio-immunodiffusion and haemagglutination, demonstrated milk-specific IgG and IgA which rose to a peak within the first few months and began to decline after the first year in infants subject to early exposure to cow's milk. Exclusive breast-feeding delayed the rate of rise of

these antibodies and reduced the peak and final levels achieved at one year, in some cases completely abolishing the response. This effect of breast-feeding on the appearance of circulating antibodies has been confirmed by others using more modern ELISA techniques,[11,12] and has been observed to be paralleled by a similar effect on numbers of circulating cells secreting milk-specific IgA.[13] Furthermore, feeding with a hydrolysed formula containing casein peptides, and therefore presumed to be non-immunogenic, appears to have a similar effect to breast-feeding.[14] However, one study could only demonstrate such a delay for the appearance of specific IgG (but not IgA or IgM),[15] and another, which only investigated IgA and IgM, found no effect.[16]

Recently, there has been considerable interest in investigating the IgG subclass distribution of antibodies to dietary antigens, since it had been suggested that antibodies of the IgG_4 isotype might be related to food allergy (reviewed by Barnes[17]). However, Husby *et al.*,[18] in an investigation of antibodies to milk proteins (by rocket radio-immunoelectrophoresis) in healthy adults, found that all IgG subclasses, with the exception of IgG_3, were represented (although Barnes *et al.*[19] did find IgG_3 to milk, by ELISA, in 53% of normal adult sera), and that specific IgG_4 could often be detected even when total serum IgG_4 was low. Furthermore, Kruszewski *et al.*[20] demonstrated specific IgG_4 against food allergens in 92% of normal blood donors, and Roger *et al.*[21] found that its presence in ovalbumin-sensitive children correlated poorly with specific IgE, and was a poorer predictor of a positive oral provocation test. There would, therefore, appear to be little doubt that antibodies of the IgG_4 subclass are part of the normal repertoire of antibodies to dietary antigens. Notwithstanding this, the observation that antibodies to dietary antigens do not decline with age in the usual way in subjects with apparently non-diet-related allergic

conditions, has repeatedly been made.[19,22]

In summary, the development and later decline of circulating antibodies to dietary antigens appears to be an integral part of the ontogeny of the normal humoral immune response. The antibody response to specific proteins is partly determined by age of first exposure. Antibodies of all isotypes can be produced, but among the IgG subclasses, there is no consensus on whether special significance can be attached to any particular response profile.

Observations in different disease states

Diabetes. Following a reported association between early exposure to cow's milk and the onset of childhood insulin-dependent diabetes (IDDM), the possible role of an aberrant immune response to milk proteins has been investigated.

In a large and comprehensive study in Finnish diabetic children, Virtanen *et al.*[23] measured IgG and IgA antibodies, by ELISA, to cows' milk formula, BSA and β-lactoglobulin (βLG). Using multivariate analysis, IgA to milk formula was shown to be an *independent* risk factor for IDDM among both diabetic-sibling control and diabetic-population control pairs, when infant feeding patterns (duration of breast-feeding and age at exposure to cow's milk) and current milk consumption were taken into account. Among diabetic-sibling control pairs, it was also independent of the presence of islet cell antibodies. Similar results were obtained from a Swedish study, although this suggested that antibodies specifically directed against βLG were an independent risk factor.[24]

Other researchers have approached this problem using a newer technique for measuring specific antibodies — particle-concentration fluoroimmunoassay (PCFIA).

Karjalainen *et al.*[25] found that this could detect IgG anti-BSA among newly diagnosed diabetics with 100% sensitivity and 98% specificity, and that this subset of antibodies was not demonstrable by standard ELISA, whereas both techniques could detect increased IgA anti-BSA in diabetics. These findings were extended when it was shown that a large amount of the anti-BSA activity in diabetics was inhibitable by ABBOS, a 17-mer peptide having cross-reactivity with a 69 kD β-islet cell surface protein, p69.[26] These antibodies appeared to return to normal within 2 years of diagnosis, after which most of the remaining anti-BSA was not inhibitable by ABBOS. These results were supported by a French study which detected IgG anti-BSA by PCFIA in new diabetics with a disease sensitivity of 74.4% and specificity of 95%, compared with 83.7% and 98%, respectively, for islet cell antibodies.[27]

Coeliac disease. Coeliac disease (gluten-sensitive enteropathy) is the paradigmatic example of immunologically mediated enteropathy,[28] and, as such, it will be discussed in greater detail elsewhere. It is characterised by the presence of at least two classes of circulating antibodies (particularly, of IgA isotype): *i)* antibodies specific for alcohol-extractable components of wheat gluten — anti-gliadin antibodies (AGA),[29] and *ii)* autoantibodies to a component, or components, of connective tissue, classified as anti-[R_1 reticulin], anti-endomysial (AEA) and anti-jejunal antibodies. The latter three may well be identical, although this has not been proven, but they are not the same as AGA, since, unlike AGA, their activity is unaffected by absorption with gliadin.[30] Moreover, AEA are more disease-specific, and therefore have a higher positive predictive value, than AGA.[31–33] However, although AGA and AEA do not appear to be cross-reactive, explants of small intestinal mucosa have been shown to produce AEA on stimulation with gliadin,[34] and it is possible that this is due to

sharing of T-cell epitopes between antigens with distinct B-cell epitopes. Finally, it should be noted that none of these antibodies are likely to be pathogenic, since subjects with selective IgA deficiency are at increased risk of developing coeliac disease.[35]

Notwithstanding the special significance of AGA, patients with coeliac disease have been demonstrated to have increased antibodies to other dietary antigens. For example, Taylor *et al.*[36,37] found highly significant increases in haemagglutinating antibodies to casein, αLA and βLG (as well as gluten fraction III — a water-soluble peptic/tryptic digest of gluten) in coeliacs, who differed from the normal controls more markedly than any of the other disease groups examined. Although these authors could not detect precipitating antibodies, these were demonstrated by Ferguson and Carswell,[38] who found an increased prevalence of serum precipitins to wheat, gluten, oats, milk, bovine and sheep serum, and egg yolk (but not rice, cornflour or egg white) in coeliac children; concomitantly, only gluten-specific precipitins were present in intestinal secretions. Similarly, Kenrick and Walker-Smith[39] demonstrated precipitating antibodies to at least one of a range of dietary antigens in 50% of coeliacs and none of the control group, with statistically significant results for soluble wheat extract (though, interestingly, not gliadin *per se*), whole cow's milk and whole bovine serum. Davidson *et al.*[40] found antibodies to maize, by indirect immunofluorescence, in 44% of coeliac patients, and, unlike wheat antibodies (detected in 68% on normal diet and 43% on gluten-free diet), their prevalence was not lower among those on a gluten-free diet. Using ELISA, Scott *et al.*[41] showed that antibodies to casein, αLA, βLG and ovalbumin were raised in untreated coeliac disease and found that they did revert to normal on a gluten-free

diet. Barnes and Lewis-Jones[42] reported a rather complex profile of antibodies in patients with dermatitis herpetiformis, in that IgG against gliadin, milk and ovalbumin, and IgA against milk could be detected irrespective of dietary exclusion of gluten, whereas IgA anti-gliadin was only found in patients not on a gluten-free diet.

Thus, since the causal role of dietary gluten in the pathogenesis of coeliac disease (in the presence of an appropriate immunogenetic background) is fully established, AGA can be considered to represent a special case of disease-associated antibodies to a specific dietary antigen. Although antibodies to dietary antigens unrelated to gliadin are no longer considered to be of diagnostic utility in coeliac disease,[43] they are frequently secondarily elevated, perhaps as a consequence of the increased mucosal permeability associated with the disease.[44–46]

Liver disease. Triger *et al.*,[47] using particle agglutination assays, found only equivocal evidence for raised antibodies to dietary proteins (ovalbumin, αLA, βLG and gluten fraction III) in liver disease, although there were convincing increases in antibodies to bowel commensal organisms (*E. coli* and *bacteroides* spp.) in the same patients, whether they were suffering from acute hepatitis, chronic active hepatitis or cirrhosis. However, André *et al.*,[48] using the same technique, did find raised antibodies to these antigens, and to BSA, in alcoholic cirrhotics; furthermore, the antibody levels were higher among the 50% of patients in whom circulating immune complexes could be detected post-prandially. Subsequently, Tamura *et al.*[49] found increased haemagglutinating antibodies to BSA in adults with liver disease (especially chronic active hepatitis and cirrhosis), while Lerner *et al.*,[50] by ELISA, reported antibodies to cow's milk proteins (especially, IgA anti-casein and IgG anti-BSA) in

children with various liver pathologies. Recently, Aitola *et al.*,[51] in a study of patients with ulcerative colitis, showed that IgA anti-casein, by ELISA, was higher in subjects who also had abnormal liver histology, and correlated positively with serum γ-glutamyltransferase and alanine aminotransferase.

One possible mechanism for these phenomena is that there is an enhanced systemic immune response to dietary proteins secondary to impaired hepatic clearance of antigenic material absorbed by the gut.[50]

Inflammatory bowel disease. In 1961, Taylor and Truelove[52] reported increased antibodies to casein and βLG (but not αLA or ovalbumin) in patients with ulcerative colitis. These were detectable by haemagglutination but not Ouchterlony double diffusion. None of the antibody titres correlated with age, sex, maintenance of a milk-free diet, extent or activity of disease, or steroid therapy, but anti-casein was positively correlated with the chronicity of disease. In an extension of this study,[37] they showed that antibodies to gluten fraction III were also raised in ulcerative colitis, whereas patients with Crohn's disease had either normal or markedly reduced antibody titres to the same panel of antigens. However, when Jewell and Truelove[53] re-addressed this issue a decade later, using a modified haemagglutination technique, they failed to find raised antibodies in inflammatory bowel disease, although they were elevated in coeliac disease. Falchuk and Isselbacher[54] used the Farr assay to measure antibodies to BSA, and found them to be present in 86% of Crohn's disease patients, 57% of those with ulcerative colitis and 43% of normal subjects (as well as five out of five with untreated coeliac disease), the increased incidence in disease being due to the presence of high titre antibodies. Furthermore, in the inflammatory bowel disease patients, high titres were more frequently associated with moderate to

severe, rather than mild, disease. These authors reported no difference in titres according to the site affected by Crohn's disease (however, the number of patients with only large bowel disease was very small). Using indirect immunofluorescence, Davidson *et al.*[40] found antibodies to maize and wheat in 33% and 58%, respectively, of Crohn's disease patients, 50% and 50% of those with ulcerative colitis, compared with 14% and 12% of normal individuals. Using an isotype-specific solid phase radioimmunoassay (a precursor of ELISA), Paganelli *et al.*[55] demonstrated increased levels of IgG and IgM (but not IgA) anti-βLG in both Crohn's disease and ulcerative colitis. In the Crohn's group, specific IgG was higher in the presence of small bowel disease and in those with active disease, as determined clinically or by laboratory indices of inflammation, whereas specific IgM was higher in those with normal laboratory indices. Specific IgG was also higher in untreated Crohn's patients, although there was no correlation with duration of symptoms or time since diagnosis or presentation. Knoflach *et al.*[56] measured isotype-specific antibodies to five cow's milk proteins (α-casein, βLG-A and -B, αLA and BSA) by ELISA — IgM to all five was raised in both ulcerative colitis and Crohn's disease, IgG to all five in ulcerative colitis, IgG anti-casein in Crohn's disease, and IgA anti-βLG in ulcerative colitis and Crohn's disease. In the Crohn's disease group, there was significant correlation between serum α_1-acid glycoprotein (orosomucoid, AAG) and IgG anti-casein, IgA anti-casein and IgA anti-BSA. By contrast, in a very similar study in chidren and young adults, Lerner *et al.*[57] found raised IgG to βLG-A, βLG-B and BSA (but not αLA or casein) in Crohn's disease (but not ulcerative colitis), raised IgA anti-casein in both Crohn's disease and ulcerative colitis, and no significant changes for specific IgM. In this study, IgG anti-BSA was higher in those with more active Crohn's

disease, but there was no correlation with treatment, duration or site of disease, and no difference between sexes. Recently, Song *et al.*[58] reported increases in antibodies to bacterial and food antigens in Crohn's disease and ulcerative colitis, but with a predilection for Crohn's disease.

As with coeliac disease, it is possible to hypothesise that increased permeability of the gut mucosa to macromolecules may underlie observations of an enhanced systemic humoral immune response to dietary antigens in inflammatory bowel disease. It may also be of relevance that permeability changes are more readily detected in Crohn's disease, and appear to be more marked in active disease.[44,59,60]

Other conditions. Other clinical circumstances which have been reported to be associated with the presence of antibodies to dietary antigens include oral ulceration,[37,61] sickle cell anaemia,[62] cystic fibrosis,[63] Down's syndrome,[64] rheumatoid arthritis[65] and IgA nephropathy;[66] in the latter, increased intestinal permeability was also demonstrated.

One of the more interesting associations is with selective IgA deficiency. This is the commonest of the humoral immunodeficiency syndromes, with a prevalence in the U.K. of ~1 in 700. Although the majority of cases are thought to be clinically silent, the incidence of autoimmune disease (including coeliac disease and connective tissue diseases) and allergy is increased in this population.[67] Buckley and Dees[68] found precipitating antibodies to cow's milk in 18 of 24 subjects with IgA deficiency, compared with 2 of 65 normal control subjects; the IgA-deficient group also had higher titres of haemagglutinating antibodies. Huntley *et al.*[69] went on to provide evidence that these precipitins were primarily directed against bovine IgM, and were cross-reactive with IgM from a variety of ruminant species (goat, sheep, deer and

elk). Cunningham-Rundles *et al.*[70] showed that the presence of milk-specific IgG, detected by ELISA, in IgA-deficient subjects, correlated with the presence of precipitating antibodies and the appearance of circulating immune complexes after drinking milk. These observations are in keeping with the view that, under normal circumstances, secretory IgA may have a role in attenuating the systemic immunogenicity of dietary antigens.

CELLULAR RESPONSES TO DIETARY ANTIGENS

Historically, the study of *in vitro* cellular responses to food-derived proteins has received less attention than that of specific antibodies. However, more widespread adoption of techniques in cellular immunology has lead to a change in emphasis in recent years. Again, much of the data which are available have been derived from work done in the context of clinical conditions putatively related to food hypersensitivity.

Food allergy. Borrone *et al.*[71] were the first to report enhanced cellular responses to a dietary antigen in this context. They found positive responses to milk protein in 10 of 21 children with chronic diarrhoea associated with milk consumption and in 3 of 3 with "milk allergy" (which they did not define). May *et al.*[72] examined cellular responses to milk protein, ovalbumin, wheat and soy in patients with type I hypersensitivity reactions to foods; although their results were somewhat equivocal, they did show that positive responses were possible in asymptomatic control subjects, whose cells gave mean stimulation indices (SIs) of 1.94 and 3.44 to soy and milk, respectively. Scheinmann *et al.*[73] obtained positive responses to αLA and/or βLG in 37.8% of subjects with immediate or delayed symptoms associated with milk

consumption (these groups were incompletely differentiated in this study), and in 9.3% of healthy subjects, and also found that the prevalence of a positive response was higher in those in whom the onset of symptoms had been in the first month of life. More recently, Eigenmann *et al.*[74] reported that lymphocyte responses to milk proteins in children with milk allergy were no different from those of normal controls, although the response to a hydrolysed formula feed was lower.

Van Sickle *et al.*[75] specifically investigated patients with milk and soy protein-induced enterocolitis and correlated the results of antigen-specific lymphocyte stimulation assays with oral challenge tests. They found geometric mean SIs of <2.2 to all antigens when the oral challenge test was negative; when the relevant oral challenge test was positive, the geometric mean SI was 8.5 to soy, and 6.0 to casein (SIs to whey protein were lower at 3.3). These investigators considered an SI of 3.6 to be the upper limit of normal for asymptomatic subjects.

Kondo *et al.*,[76] working on the basis that atopic dermatitis (eczema) macroscopically and histologically resembles a cell-mediated (type IV hypersensitivity), rather than antibody-mediated, immune response, investigated the proliferative response of peripheral blood mononuclear cells (PBMC) to BSA and ovalbumin in chidren with this condition who had delayed onset cutaneous symptoms following ingestion of milk or egg. They found mean maximal stimulation indices of >3 to the relevant antigen in eczema, compared with <1.5 in normal controls and chidren with immediate hypersensitivity (urticaria/angioedema) to milk or egg, the difference being highly statistically significant. The prevalence of positive antigen-specific IgE (RAST score >1) in the eczema group was less than half that in the immediate hypersensitivity group; furthermore, there was no correlation between

RAST score and the lymphocyte stimulation index. These investigators went on to show that the proliferating cells were $CD4^+$ T-cells, and that antigen-presenting cells were necessary for the response. Furthermore, following an elimination diet, symptomatic improvement was accompanied by a decrease in the cellular response which was specific for the excluded antigen. This group has also examined the cytokine secretion profile of cells proliferating in response to ovalbumin. Proliferation in egg-sensitive eczema was accompanied by production of IL-2 and IFNγ, and the latter distinguished patients from normal controls and those with immediate hypersensitivity. In all patients, there was inverse correlation between IFNγ and IgE production, and positive correlation between IL-4 and IgE.[77–83] The conclusion of these authors was that food-sensitive eczema is associated with an antigen-specific T-cell response resembling the pro-inflammatory Th_1 phenotype, in contrast to the Th_2-like response of T-cells which give rise to type I hypersensitivity. A Th_2-like cytokine profile of ovalbumin-specific T-cells isolated from egg-allergic individuals with eczema *and* antigen-specific IgE has recently been described by Katsuki *et al.*[84]

In the hands of these researchers, the ability of the lymphocyte stimulation assay to differentiate subjects with immediate from those with delayed hypersensitivity is impressive. Although the virtual unresponsiveness of cells from normal control subjects is unusual in the context of other studies on dietary antigens cited in this section, a similar pattern has been described for cellular responses to *Dermatophagoides pteronyssinus* (house-dust mite) allergen, *i.e.* negligible stimulation of cells from normal donors and maximal responses in the presence of atopic dermatitis.[85] If reproducible, these results demonstrate the importance of the accurate classification of subject groups according to clinical criteria, since they imply that T-

cell responses may depend qualitatively and quantitatively on the precise clinical manifestations of different hypersensitivity syndromes related to atopy. There is one report which suggests that responding T-cells in milk-induced eczema express a skin-specific homing receptor — cutaneous lymphocyte antigen (CLA)[86] — and it is possible that lymphocyte subsets which preferentially home to different anatomical sites are unequally represented in peripheral blood.

Type I hypersensitivity to nuts is a clinically significant cause of anaphylaxis, and peanuts are currently the major cause of fatal attacks due to food in the U.K. (R.S.H. Pumphrey, personal communication). Two groups have recently isolated peanut-specific $CD4^+$ T-cell clones from allergic patients and shown that these preferentially produce Th_2 cytokines. Higgins *et al.*[87] found positive proliferative responses to peanut, hazelnut and Brazil nut in allergic patients and normal controls (SIs were markedly higher than those obtained in the studies discussed above), with a tendency to higher responses in the allergic group; this difference was statistically significant in the study of de Jong *et al.*[88] Dorion and Leung[89] have claimed that, following antigen-specific stimulation of cells from peanut-allergic subjects, there is expansion of T-cells with receptors incorporating the $V\beta_2$ variable region.

Diabetes. Data on cellular responses to milk-derived proteins in diabetes are conflicting and differ along the same lines as the antibody data which have been discussed — one group of workers has found T-cell responsiveness to the BSA-derived ABBOS peptide in 28 of 31 children with newly-diagnosed IDDM but not in controls,[90] whereas another group found positive responses to βLG (but not BSA or casein) in 55% of IDDM compared with 22% of controls.[91]

Coeliac disease. Although the exact mechanism by which the gastrointestinal lesion

of coeliac disease is generated is not yet known, there is a great deal of evidence to suggest that gliadin-specific T-cells are instrumental in the disease process.

Coeliac disease is very strongly associated with the presence of a particular MHC class II heterodimer, DQ2, encoded by the alleles DQA1*0501 and DQB1*0201 in either *cis* or *trans* position,[92] and the peptide motifs necessary for binding to this molecule are known.[93–96] The same association with DQ2 is also present in patients with dermatitis herpetiformis.[97] Gliadin-specific $CD4^+$ T-cells isolated from the intestinal mucosa of patients with coeliac disease have been found to be preferentially restricted by DQ2,[98] and parallel observations have been made for the subset of coeliac disease patients in whom the disease-associated class II molecule is DQ8, encoded by DQA1*0301/DQB1*0302.[99] Furthermore, a DQ2-restricted, peripheral blood-derived T-cell clone from a coeliac patient was found to recognise a peptide comprising residues 31–47 of α-gliadin,[100] which has also been shown to be toxic *in vivo*.[101] However, the restriction elements for blood-derived T-cells responding to gliadin are more diverse than for mucosa-derived cells[102,103] — indeed, one group found only DR-restricted cells in blood from a patient and a normal subject (interestingly, the peptides being recognised were not those thought to be associated with disease).[104]

Gluten-specific T-cells, whether from blood or mucosa, or from healthy or coeliac subjects, and regardless of restriction element, consistently appear to produce IFNγ on antigenic stimulation, and have been classified as Th_0 or Th_1 cells.[105,106] They are able to mediate enterocyte damage *in vitro*, an action which is mimicked by IFNγ and can be blocked by anti-IFNγ.[107] IFNγ mRNA (by *in situ* hybridisation) has been found to be increased in the gut mucosa in untreated coeliac disease, compared with

coeliacs on a gluten-free diet and normal subjects.[108] In view of the detailed information available on the importance of T-cell sensitisation to gluten in coeliac disease, the relatively low SIs (mean of ~2, *c.f.* 1 in controls) to gluten, reported by Sikora *et al.*,[109] are disappointing.

Although gluten-specificity of intra-epithelial lymphocytes (IELs) has not been established, the number of these cells is increased in coeliac disease, and the view is emerging that they may be the ultimate mediators of mucosal damage, possibly driven by cytokine(s) released by antigen-specific lamina propria lymphocytes (LPLs).[110] This view is supported by the high granzyme B content of IELs in coeliac disease, compared with normal subjects and those with giardiasis.[111] Differential expression of activation and proliferation markers on IELs and LPLs has been described,[112,113] and it is possible that cytotoxic IELs proliferate in response to non-proliferative activation of LPLs.[114] A high proportion of IELs express γδ T-cell receptors, and increased numbers of circulating γδ cells have been reported in children with coeliac disease.[115] Although this was not confirmed in a study of adults, increased frequency of expression of CD45RO on circulating lymphocytes was demonstrated. This was attributed to $V\delta_1$-$J\delta_1$ cells, which, although decreased in number (possibly due to sequestration in the gut), were more frequently of this 'memory' cell phenotype than the equivalent population in healthy individuals.[116] The preferential use of other Vγ and Vδ T-cell receptor genes has been reported in coeliac disease,[117,118] but the significance of this is not known.

Inflammatory bowel disease. In one published study of lymphocyte responses to dietary antigens in Crohn's disease — a comparison of 11 Crohn's disease patients with 9 normal controls — SIs to milk were statistically significantly increased (3.6

c.f. 1.4) in the disease group and there was a similar, but not significant, trend for egg, wheat and soy.[119]

SUMMARY. Antibody responses to dietary antigens occur during the course of normal development and are age-dependent. Quantitative and qualitative differences occur in the presence of certain disease processes. In at least some of these, enhanced antibody production might be attributable to altered permeability of the gastrointestinal mucosa or impaired clearance of antigen. In others, there may be a genetically determined predisposition to sensitisation to specific protein(s).

Cellular responses, as represented by proliferation of peripheral blood lymphocytes, to dietary antigens also occur in normal individuals but there is considerable variation between studies and between responses to different proteins. There is, therefore, no consensus as to the expected magnitude of these responses in the normal population. Atopic eczema with food sensitivity is perhaps the best example of a pathology associated with a measurably increased response in peripheral blood. However, the underlying mechanism of T-cell sensitisation to a specific dietary antigen is best-established in the case of coeliac disease.

Yeasts as antigens

Of yeasts relevant to human health, the commensal and opportunist pathogen, *C. albicans*, has been the most extensively studied with respect to the nature of the immune response elicited by it. However, in studies of the structure of the molecular species implicated as dominant antigens of *C. albicans*, *S. cerevisiae* has often been used as a model. Experimental data on immune responses to yeasts can be more

easily interpreted in the light of information on the chemical composition of cell wall mannoprotein; therefore, this will be considered first. Much of the information in this section is comprehensively reviewed in refs. 120–122.

ANTIGENIC STRUCTURE OF MANNAN

Cell surface determinants which define serotypic differences between yeasts are mainly, and perhaps exclusively, located on the structural mannans (mannoproteins, peptidomannans), which constitute 20–23% of the cell wall, by weight.[123,124] In *S. cerevisiae* and *C. albicans*, the mural mannan consists of a structural protein to which mannose polymers are covalently bound by two different glycopeptide linkages:

i) N,N'-diacetylchitobiosylaspartamido (glycosylamine) bonds, by which linear polysaccharides, with up to ~30 mannose residues in $\alpha 1 \rightarrow 6$ linkage, are attached to asparagine residues *via* *N*-acetylglucosamine — these are said to be '*N*-linked' and recapitulate a joining sequence present in mammalian glycoproteins;

ii) hydroxyamino acid ester bonds, by which short oligosaccharides, with up to 4 mannose residues in predominantly $\alpha 1 \rightarrow 2$ linkage, with some $\alpha 1 \rightarrow 3$ terminal residues, are attached to serine or threonine — these are said to be '*O*-linked' and have no mammalian counterpart.

In addition, the *N*-linked $\alpha 1 \rightarrow 6$ polymannoside is further divided into an 'inner core' and an 'outer chain'; these are both substituted with oligomannoside side-chains similar to those *O*-linked to the protein, but only those attached to the outer chain have side-branches linked by phosphodiester bonds. Following ion exchange

chromatography on diethylaminoethyl (DEAE) Sephadex, reactivity with antisera against the parent yeast can be co-purified with mannan fractions with a high phosphate content; therefore, variation in these *O*-phosphonomannans may be largely responsible for serologically determined antigenic differences between species and strains, *i.e.* they appear to contain major B-cell epitopes[125] (there is firm evidence for this in the case of *C. albicans* group B[126]). The obligate presence of a protein component in mannan (which, depending on the method of purification, may be up to 10% of the total weight[127]) testifies to the possibility that T-cell epitopes may be present in the same molecule.

Mannans obtained from any given source are heterogeneous both with respect to molecular weight[128] and charge.[129] In addition, they can be altered by the method of purification: for example, the alkaline conditions associated with precipitation with Fehling's solution result in a lower molecular weight product (~40 kD)[130] due to loss of *O*-linked oligomannosides and cleavage of some peptide bonds — this product, when obtained from *C. albicans*, will bind antibody raised against crude extracts of yeast cell wall but not stimulate its production (*i.e.* it is antigenic but not immunogenic);[123,131] nor is it an efficient stimulator of proliferation in lymphocyte cultures (an observation partly, but not completely explained by interference from residual bound copper).[132,133] In contrast, mannan precipitated with cetyltrimethylammonium bromide (cetavlon, CTAB) retains a high molecular weight (~133 kD), is immunogenic and can efficiently induce *in vitro* lymphocyte proliferation (see below).

IMMUNOLOGICAL STUDIES

It is a long-standing observation that crude extracts of soluble material derived from *C. albicans* have the ability to stimulate *in vitro* lymphocyte proliferation.[134–136] Because of the known importance of mannan as the dominant antigen in humoral immune responses to yeasts, speculation arose as to whether it might also be the agent responsible for these cellular responses. On investigation, it became clear that, even though different mannan-containing preparations might react equally with pre-formed antibody, they varied greatly in their ability to *induce* an antibody response and to effect lymphocyte proliferation. For example, Gettner and MacKenzie[137] found mannan precipitated with Fehling's solution to be a very poor stimulator of lymphocyte proliferation unless it was adsorbed on to latex particles. Durandy *et al.*[138] absorbed the same mannan preparation with methylated bovine serum albumin, and showed that this rendered it able to induce a lymphoproliferative response and to stimulate *in vitro* T-cell-dependent production of antibody which was reactive with the unabsorbed mannan. Wirtz *et al.*[139] found that a 'partially purified polysaccharide fraction' (MPPS), extracted under mild conditions, induced lymphocyte proliferation, whereas a more degraded mannan preparation did not, even though they competed for binding of rabbit anti-*Candida* antibody.

Piccolella *et al.*[140] co-cultured T-cells (as sheep erythrocyte-rosetting, E^+, cells) and B cells (as non-rosetting, E^-, cells, further depleted of monocytes by adherence) in the presence of MPPS, and showed that the resulting proliferative response was sensitive to mitomycin C treatment of the T-cell component, whereas similar treatment of the B-cell population left the response relatively intact. They went on to demonstrate a requirement for cells expressing Ia antigen (*i.e.* MHC class II),[141] and

that this function could be subserved by monocytes/macrophages[142] (although they had previously considered only B-cells to be capable of this).

Ausiello *et al.*[143] also demonstrated differential lymphocyte responses to various mannan preparations. One of these — a neutral citrate buffer extract, termed GMP (probably identical to MPPS), containing mannose as 98.4% of the total polysaccharide, with 8% protein — was particularly active. It was found to stimulate uptake of tritiated thymidine in peripheral blood mononuclear cells but not umbilical cord blood mononuclear cells. Together with the inhibition of proliferation by an anti-DR monoclonal antibody, this was taken to imply antigen-specificity of the T-cell response. GMP was also shown to induce non-MHC-restricted cellular cytotoxicity in mice[144] and man.[145]

Torosantucci *et al.*[146] subjected GMP to pronase digestion, which abolished its lymphocyte-stimulating ability but preserved its antibody-binding capacity. The lymphoproliferative response was also abolished by a non-mitogenic anti-CD3 monoclonal antibody. On fractionation by ion-exchange chromatography, the lymphocyte-stimulating capacity of GMP co-separated with a fraction containing a high molecular weight band (>200 kD) on SDS-PAGE. The composition of this fraction, which constituted 47.6% of the original material, was >90% mannose and 5% protein.

Podzorski *et al.*[133] performed lymphocyte proliferation assays with mannans prepared by precipitation with Fehling's solution or CTAB, and demonstrated that CTAB precipitation preserved immunological activity which was partially lost as a consequence of Fehling's precipitation, confirming the importance of the native structure of mannan to its effectiveness as a T-cell antigen.

Durandy *et al.*[147] found evidence for predominant DQ restriction of the T-cell response to mannan. However, the preparation used in this study was the methyl-BSA/Fehling's precipitated mannan previously used by this group (see above), and confidence in this finding would be enhanced if it could be reproduced with an unmodified mannan preparation.

SUMMARY. Yeast cell wall mannans are the immunodominant antigens in humoral immune responses to these organisms. Mannans are similar in overall chemical composition, but differences in fine structure determine the species- and strain-specificity of the antibody response. Antibody responses to mannans are T-cell-dependent. T-cell responses to mannans are classical antigen-specific responses which require antigen-presenting cells, are MHC-restricted, and depend on the relative intactness of the protein component of the mannan.

Notes on techniques

Measurement of antibodies

As discussed above, studies of antibodies to dietary antigens have often yielded conflicting results. No doubt, this is partly due to sampling error, but the wide variety of techniques used is likely to have been a major contributor. The earliest antibody assays had end-points which relied on some measure of secondary manifestations of antibody-binding, such as haemagglutination, precipitation or complement activation. These depend on functional characteristics of immunoglobulin molecules which differ between isotypes. Primary binding assays measure bound antibody regardless of biological function and they are also more sensitive. For

example, McCaffery *et al.*[148] compared the Farr assay with haemagglutination and gel precipitation for the measurement of anti-BSA in patients with inflammatory bowel disease and healthy controls — the Farr assay detected anti-BSA in 23% of controls, 42% of Crohn's disease subjects and 37% of those with ulcerative colitis, gel precipitation (micro-Ouchterlony double diffusion) was negative in all cases, and haemagglutination was positive in less than half of the samples with a high binding capacity in the Farr assay. Furthermore, it is clear that in comparisons between groups, different results might be arrived at depending on whether the data being compared are based on *prevalence* (all-or-nothing, descriptive, response) or *magnitude* (numerical response).

The use of ELISA for antibody measurement was first described by Engvall and Perlmann,[149] and rapidly gained widespread acceptance in a variety of applications for assessing serological responses in infectious diseases.[150] Apart from the introduction of disposable microplates to replace tubes as solid phase supports,[151] the basic technique remains largely unchanged since its original description. A major advantage of ELISA is that it allows accurate standardisation.

Previous attempts to measure serum antibodies to *Saccharomyces cerevisiae* have taken absorbance alone as the end-point of the assay, or have relied on the concept of a 'binding index'. Although both of these approaches represent valid attempts to produce quantitative data, there are significant objections to them. Firstly, neither allows discrimination between samples giving absorbance values at or near the upper end of the range which is measurable (or, in practice, encountered under the conditions of the assay). Secondly, even if inter-assay standardisation is attempted by kinetic monitoring of the colour development reaction and taking all the readings

when a particular reference well (or wells) has achieved a predetermined absorbance value, it cannot be assumed that the shape of the (implied) titration curve described by the reaction will be constant each time the assay is performed; in effect, this is a single-point calibration.

In order to obviate these problems in the experimental work described here, ELISAs were developed in which the antigen-binding activity of test sera could be derived from calibration curves generated by serial dilutions of constant standard sera. Since a new curve is created each time the assay is performed, it is internally controlled for variations in conditions, such as temperature and incubation times. Furthermore, test samples giving absorbance values occupying the upper plateau of the calibration curve can be correctly quantified after further dilution. The practical considerations inherent in this approach have been discussed.[152]

LYMPHOCYTE CULTURE

The ability to establish cultures of lymphocytes *in vitro* has been fundamental to the development of current knowledge of basic immunological processes. However, many potential sources of error exist when these techniques are used to derive quantitative data, and it is worth considering these in the context of the historical development of the various methods which have been used in this thesis. (A very informative review of the early history of lymphocyte culture is contained in reference 153.)

Proliferation assays. In effect, it was the discovery (more correctly, rediscovery) of the haemagglutinating effect of an extract of the red kidney bean *Phaseolus vulgaris*[154] which was the starting point for the development of cellular immunology.

Use of this *phytohaemagglutinin* (PHA) to facilitate the preparation of leucocyte-rich cell fractions from blood or marrow led to the fortuitous observation that it appeared to stimulate mitosis in a proportion of the leucocytes thus obtained.[155] Before this, it had been uncertain how much, if any, capacity for division was retained by cells in the peripheral blood, and the first practical use made of this phenomenon was in the production of cellular preparations for chromosome analysis.[156,157] However, since the proliferating cells appeared to be the small lymphocytes,[158] whose central role in the generation of immune responses was now being realised, it was clear that the ability of any agent to stimulate these cells *in vitro* was of great physiological interest *per se*. It was soon evident that other substances also demonstrated the property of 'mitogenicity' in lymphocyte cultures — first shown for purified protein derivative (PPD) of *Mycobacterium tuberculosis*,[159] this observation was rapidly extended to many further examples, including other microorganisms and their products (diphtheria toxoid, pertussis, smallpox and polio vaccines, streptolysin O, filtrates of *E. coli*), allergens (grass pollen, ragweed, alternaria) and drugs (penicillin, aspirin, phenytoin and others).[160–169] Significantly, there were three important differences between the response to PHA and those to the great majority of other substances tested: it occurred earlier, involved a much greater proportion of the cells present at the start of the culture, and it did not depend on prior sensitisation of the subject.[160,161,170,171] It was, therefore, suggested that the mechanism of action of PHA might differ from that of 'specific' antigens; the fact that its mitogenic activity could be abolished by absorbing out its leucoagglutinating activity[161] lent weight to the suggestion that it might act by binding to surface moieties on the lymphocyte in a way analogous to its cross-linking of red cell surface polysaccharides.[172] Apart from its value to the

study of fundamental immunological processes, the polyclonal T-cell response to PHA was also found to be of some use in the investigation of primary and secondary cellular immunodeficiencies,[173–177] and, although a very non-specific test, it continues to be applied in this clinical context.

A major difficulty encountered in early studies was the problem of *quantitation* of proliferative responses, which initially had to be assessed subjectively on the basis of direct visualisation of morphological changes (such as '% blastic transformation' and numbers of mitotic figures). Even after allowing for sampling and counting errors, the inferences to be taken from microscopic observation were not always obvious — for example, the apparent percentage blast transformation in a culture will depend not only on the proportion of the original cells transformed but also on the amount of cell proliferation and death.[178] Although some attempts were made at standardisation of morphological data,[179] considerable simplification resulted from the use of radio-labelled DNA precursors, whose incorporation into cellular DNA, during a timed incubation, could be readily estimated, either directly, on washed, dried cells, or following chemical extraction with trichloroacetic acid. Of the several agents employed — [^{14}C]-thymidine,[180] [^{3}H]-thymidine,[178] [^{125}I]-iodo-deoxyuridine[181] and [^{32}P]-sodium orthophosphate[182] — tritiated thymidine became established and is in current use.

The impetus for the development of further methodological improvements, which aimed to optimise the sensitivity, reproducibility and ease of execution of the technique, followed the discovery of *in vitro* alloreactivity — the observation that lymphocytes from different individuals were co-stimulated in mixed cultures,[183] and that these data correlated both with the clinical survival of tissue grafts[184] and with

the degree of histocompatibility assessed serologically.[185] The major advance of relevance to modern practice was the replacement of 'bulk' cultures, carried out in tubes,[186–188] by miniaturised, semi-automated techniques which allowed multiple assays to be performed in disposable plastic multi-well plates, the final product being harvested directly on to glass fibre filters before β-counting in liquid scintillant.[189–197] Although some groups have favoured the use of very small volume cultures (~20 μl) in the wells of Terasaki plates,[198–209] and have produced impressive results with that system, it has not been universally adopted, probably because it is more technically demanding. Currently, quantitative lymphocyte proliferation assays are almost universally carried out in 96-well polystyrene plates, which is the technique used in the work reported here. In addition, isolation of mononuclear cells, with minimal erythrocyte or granulocyte contamination, was made easier and more reliable with the introduction of density gradient centrifugation techniques.[210]

It is well documented that these assays are very sensitive to variations in virtually any of the conditions under which they are carried out; it is important that this is borne in mind when attempting to derive quantitative information from them, and that every effort is made to standardise as much of the procedure as possible.

Precursor frequency analysis. Although the techniques described above have proved valuable in permitting qualitative and semi-quantitative measures of certain aspects of immune function, there is such variability in the exact magnitude and timing of responses within and between experiments that it is not possible, under any given conditions, to assess what proportion of the cell population (the *precursor frequency*) present at the initiation of the culture is responding to the applied stimulus. In order to address questions of this nature, the method of *limiting dilution analysis* (LDA) has

been applied.[172,211–213] The theoretical basis of LDA is that inferences can be made from statistical analysis of culture systems in which one or more of the participating cell types are present in *limiting* amounts — *i.e.* they are so infrequent that a measurable proportion of 'replicate' cultures will receive *no* cells of that type. In its simplest form, only one cell type is limiting and all other components of the culture are non-limiting, *i.e.* available in sufficient excess as to render the experimental conditions fully permissive to the limiting cell type, allowing the original presence of as few as one of these in the initial culture to be detected by some suitably sensitive end-point assay (*e.g.* [^{3}H]-thymidine uptake, cytotoxicity, antibody or cytokine secretion).

If a number of 'replicate' cultures are established in which the limiting cell type is present at a *mean* frequency of μ cells/well, then the probability, P_x, of any well containing x cells is defined by the Poisson probability distribution[214] as:

$$P_x = \frac{\mu^x e^{-\mu}}{x!}$$

This probability equates to the expected value of the fraction, F_x, of the total number of wells in the experiment which originally contained x of the limiting cell type. Because the end-point measurement is an all-or-nothing response (*i.e.* wells are either 'positive' or 'negative', but the initial number of responders in any positive well is not known), only the fraction of wells for which $x = 0$ — the 'negative' wells — can be directly determined experimentally. From the zero term of the Poisson distribution:

$$F_0 = e^{-\mu}$$

Now, $\mu = \phi c$, where ϕ = the proportion of cells of the limiting type in the sample being tested, and c = the mean number of sample cells per well (often called the cell

input), therefore:

$$F_0 = e^{-\phi c}$$

$$\Rightarrow -\ln F_0 = \phi c$$

Thus, a plot of $-\ln F_0$ (in practice, F_0 on a log scale) against c will yield a straight line through the origin; the unknown precursor frequency, ϕ, can be calculated from the gradient, or from the value of 1/c when $F_0 = 0.37$ (*i.e.* $\mu = 1$ — there is an average of one limiting cell per well at that value of c).

This is the situation when the system conforms to a *single-hit* model, where only one limiting cell in a well is necessary and sufficient to give a positive result. It is necessary to derive F_0 at different values of c in order to assess the 'goodness-of-fit' of the experimental data to the single-hit model — any significant departure from linearity suggests the possibility of a more complex system, *e.g. multi-hit* (two or more of the same cell type necessary for a response), or *multi-target* (two or more different cells necessary). The relative suitability of different statistical methods of data-fitting for the calculation of precursor frequency (minimum chi-square,[215] maximum likelihood,[216] jacknifed maximum likelihood[217]) and for analysing goodness-of-fit (chi-square,[215] log likelihood and modified Weibull[218]) has generated some debate, but it has been asserted that uniformity of approach is the most important factor for obtaining meaningful comparisons.[212]

Examples of LDA, using an end-point of T-cell [^{3}H]-thymidine uptake, include measurements of the frequency of circulating specific precursors responding to PPD, tetanus toxoid, *M. leprae* and *Leishmania*,[219–221] and of precursors in umbilical cord blood recognising mycobacterial 65 kD heat-shock protein.[222] LDA is currently being explored as a method of assessing the potential for clinically significant alloreactivity

in the context of bone marrow transplantation.[223,224]

Long-term culture and T-cell cloning. Although it had been possible to chronically establish certain B lymphoblastoid cells as a consequence of their transformation with Epstein-Barr virus, the earliest long-term cultures of T-lymphocytes were restricted to examples of neoplastic cell lines and alloreactive cells maintained by repeated stimulation with the relevant allogeneic cells. Despite the ease with which fresh PBMC demonstrated an initial proliferation in response to mitogens and soluble antigens, simple attempts to restimulate with these agents were ineffective.

The solution to this *impasse* was provided by the observation that growth of normal T-cells from marrow or blood could be initiated and prolonged by supplementation with conditioned medium from other cultures of PHA-stimulated cells.[225,226] Kurnick *et al.*[227] using conditioned medium in this way, were able to maintain long-term growth of cells following initial stimulation with mitogen (PHA) or antigen (PPD or allogeneic cells). Significantly, the growth-maintaining properties of the conditioned medium were not diminished (in fact, were enhanced) by removal of the PHA content, a manipulation which did, however, render the medium incapable of *initiating* growth in unstimulated cells. Thus, the presence of an active principle quite separate from the contaminating lectin was established — originally designated *T-cell growth factor (TCGF)*, it is now recognised as IL-2. When the initially antigen-stimulated cells were grown in conditioned medium and later tested in proliferation assays, they were shown to have retained their specificity. Furthermore, whereas allogeneic cells were a sufficient stimulus for the alloreactive cell line, the cells with specificity for soluble antigen (PPD) required the addition of fresh syngeneic cells in order to proliferate (in contrast, the response to PHA of cells from long-term culture

could be restored with syngeneic *or* allogeneic cells). Subsequent studies confirmed the importance of MHC class II molecules as restriction elements for responses to soluble antigen.[228]

The ability to selectively enrich cell populations for a particular antigen specificity allowed the use of limiting dilution cultures for the cloning of T-cells of predetermined specificities.[229–232] Clones have proven especially useful in the investigation of MHC restriction of T cell responses because alloreactive cells have been selected out of the population,[233,234] thereby unmasking the unreactivity of certain histoincompatible MHC/antigen combinations and allowing detailed scrutiny of MHC cross-reactivity. Conversely, allospecific clones can be selected for and investigated in similar manner.[235,236] T-cell clones specific for a wide variety of antigenic substances with relevance to clinical practice[237–239] are a useful tool for investigating the immunological basis of disease and may suggest rational approaches to treatment.

I

Observations on sacc-specific antibodies in serum

MATERIALS AND METHODS

Development of quantitative ELISAs

PREPARATION OF COATING ANTIGENS

Sacc. An aqueous extract of a commercial preparation of *Saccharomyces cerevisiae* was prepared as follows:

200 g dried bakers' yeast *(J. Sainsbury, plc)* were rehydrated by suspending in 1 l pre-cooled (4 °C) distilled water, with constant mixing. The suspension was centrifuged at 2,000 rpm (~800 × *g*) in a Beckman TJ-6 centrifuge, the supernatant discarded, and the yeast resuspended to the original volume, the process being repeated for a total of three washes. The final suspension was heated to 100 °C for 1 hour in a boiling water bath and then cooled to 4 °C. After centrifugation, the supernatant was retained, filtered to 0.45 μm, dialysed extensively against distilled water and lyophilised. The resulting pale yellow-brown powder (sacc) was stored at room temperature.

Casein. Dried bovine milk casein was obtained from a commercial source *(Sigma)*.

DETERMINATION OF OPTIMAL ANTIGEN CONCENTRATION

In order to determine appropriate concentrations for the antigen solutions used to coat ELISA plates, experiments were conducted using a 'checquerboard' design. ***Sacc.*** Solutions of sacc, in doubling dilutions from 4 μg/ml to 1.95 μg/ml, were prepared in carbonate-bicarbonate buffer, pH 9.6 (see Appendix I). Aliquots of 100 μl of each of these solutions were deposited into the wells of *Immulon 2* ELISA plates *(Dynatech)*, using two rows of wells for each dilution. The plates were then sealed with adhesive tape, agitated briefly on a plate shaker, and incubated for 24 hours at room temperature. Immediately before use, the plates were washed four times with phosphate-buffered saline containing 0.05% Tween 20 *(Sigma)* (PBS-Tween, see Appendix I). Doubling dilutions, from $1/2^5$ to $1/2^{14}$ in PBS-Tween, of normal ('negative') and hyperimmune polyclonal ('positive') rabbit serum[a] were then overlaid onto the coated plates — 100 μl of each dilution of the positive and negative sera into one well for each concentration of coating antigen — the plates resealed, agitated, and incubated for 1 hour at 37 °C. Following a further four washes with PBS-Tween, 100 μl of a $1/1{,}000$ dilution of horse-raddish peroxidase-conjugated swine anti-[rabbit immunoglobulin] *(Dako)* in PBS-Tween were added to each well, and the plates resealed, agitated, and incubated for a further hour at 37 °C. After four more washes, 100 μl of *o*-phenylenediamine (OPD) *(Sigma)*, 0.4 mg/ml in phosphate-citrate buffer, pH 5 (see Appendix I), activated with 1:5,000 v/v of 30% hydrogen peroxide *(Sigma)*, were added to each well, and the plates left at room temperature during the development of the colour reaction. The latter was stopped after 5 minutes by the addition of 100 μl of 1 M sulphuric acid to each well, and the optical density

a: these sera were prepared in-house by Mrs. S. Allen

(absorbance) at 492 nm (A_{492}) read on an ELISA plate-reading spectrophotometer *(Titertek Multiscan II)*.

Casein. A similar procedure was followed in the case of casein: dilutions of the antigen were tested at approximately semilog intervals from 100 μg/ml to 0.03 μg/ml, with hyperimmune and normal rabbit sera in doubling dilutions from 1/50 to 1/25,600.

DERIVATION OF STANDARDS

In order to produce standard antisera for calibration of the assays, individual sera were screened at a single dilution for the presence of IgG and IgA anti-sacc antibodies and IgG anti-casein antibodies. The sera used were from patients with gastroenterological disorders; 69 were screened for IgG anti-sacc, 70 for IgA anti-sacc, and 51 for IgG anti-casein.

Sacc. ELISA plates were coated with sacc antigen at optimal concentration, as described above. Sera were diluted 1/200 in PBS-Tween and the assay was conducted essentially as above, except that the peroxidase-conjugated antisera used were rabbit anti-[human γ-chain] and rabbit anti-[human α-chain] *(Dako)* at 1/1,000 dilution, for the detection of IgG and IgA isotype-specific antibodies, respectively.

Casein. ELISA plates were coated with casein at optimal concentration and sera were screened at 1/100 dilution.

Sera which gave high absorbance values were then pooled to provide a separate calibration standard for each assay.

ASSAY CALIBRATION AND CALCULATION OF TEST RESULTS

Aliquots of the IgG and IgA anti-sacc, and the IgG anti-casein, calibrants were

stored at −30 °C.

Each time the ELISA was performed, an aliquot of each of the appropriate calibrants was thawed and serial doubling dilutions made. Test sera were diluted to 1/100. The dilutions of the calibrants and test sera were then assayed using 100 µl/well in duplicate wells. The IgG- and IgA-specific peroxidase conjugates used were as described above, and, in the final form of the assays, 7 1/2 minutes were allowed for colour development in the sacc assays, and 5 minutes in the casein assay, before quenching the reaction.

Calibration curves were compiled from the absorbance values obtained at each dilution of the calibrants — mean values from duplicate wells were plotted against reciprocal dilution (on a log scale) and a sigmoid curve fitted by computer, using a graphics program *(FigP)*.

A subjective judgement was made as to the 'useable' part of the curve — where the gradient was sufficient to adequately resolve differences in concentration — and the test sample absorbance values lying within this range were transformed to equivalent dilutions of the calibrant. By correcting for the sample dilution factor, antibody concentration of the *undiluted sample* could then be expressed as a proportion (%) of that of the *undiluted calibrant*. Duplicate results were obtained for each sample, and the means taken. Test samples giving absorbance values above the upper acceptable limit at 1/100 dilution were re-tested at further doubling dilutions until a concentration value could be assigned to them.

ASSAY VARIABILITY

The variability of the assays was assessed in the course of their use in testing

the normal and pathological sera whose results are discussed below.

Intra-assay variability. This was assessed on the results obtained from a large number of consecutively tested samples. The difference between each pair of duplicate result was expressed as a proportion of their mean. The set of values thus obtained could then be described statistically.

Inter-assay variability. This was assessed by testing a small number of selected sera on several occasions. Statistical analysis was performed on the means of the duplicate values obtained from each assay. (It can be seen that a component of this variability will, in fact, be determined by intra-assay variability — the true inter-assay variability would be assessed by analysing the distribution of the mean values derived from a large number of replicates; however, the method used here reflects the inter-assay variability observed *in practice*, given that samples were always assessed in duplicate.)

Application to human sera

SUBJECTS

Venous blood samples were collected from 224 normal subjects who acted as controls; 179 of these were blood donors attending the Liverpool blood transfusion centre and the remainder were laboratory staff. Samples were also obtained from 163 patients with selected gastrointestinal disorders, most of whom were under the care of a single consultant physician. The study received ethical committee approval and the patients' permission was obtained for their inclusion.

Sera were separated and stored at −20 to −30 °C until required.

The allocation of patients to each disease category was based on the clinical

diagnosis at the time of sampling, in conjunction with a review of the case notes. Demographic data pertaining to the different groups are summarised in Table 1.

GROUP	TOTAL NO.	SEX RATIO (M:F)	AGE (median; range)
normal	224	2.3	36; 21–58 *206*
Crohn's disease	51	0.85 *50*	40; 20–76 *46*
non-specific colitis	24	0.85	61; 22–73
ulcerative colitis	41	0.71	43; 24–82 *40*
chronic liver disease	23	0.44	58; 38–77 *21*
coeliac disease	17	0.13	55; 36–72 *16*
irritable bowel	7	0.4	40; 23–56

Table 1. *Age and sex data of patients and controls. Small italics denote the number of observations in cases where data was incomplete.*

Site, activity and duration of Crohn's disease. Of the Crohn's group, 32 were identified as having small bowel disease, with or without large bowel involvement, 14 were considered to have large bowel disease only, and 1 patient had oesophageal Crohn's disease.

Disease activity was assessed by means of the *Bristol Activity Index*, as described by Harvey and Bradshaw,[240] and by measurement of serum α_1-acid glycoprotein (AAG, orosomucoid) by rate nephelometry *(Beckman Array)*. The upper limit of the normal reference range for AAG was 1.2 g/l.

An estimate was made of the likely duration of disease, based, as far as possible, on the duration of symptoms.

Treatment of inflammatory bowel disease. Potentially immunomodulatory therapy taken by patients at the time of entry into the study was documented (Table 2).

Aetiology of liver disease. There were 10 patients with alcoholic liver disease (7 of whom had frank cirrhosis), 4 with chronic active hepatitis, 5 with primary and 1 with secondary biliary cirrhosis, 2 with cirrhosis of unknown aetiology and 1 with undefined chronic liver disease.

Coeliac disease. These patients were all being maintained on a gluten-free diet.

GROUP	CORTICOSTEROIDS (oral; topical; both)	AZATHIOPRINE	5-ASA DERIVATIVE (oral; topical)
Crohn's disease *(49)*	22; 2; 1	1	20; 0
Non-specific colitis *(24)*	5; 7; 1	0	17; 0
Ulcerative colitis *(41)*	14; 8; 1	2	35; 1

Table 2. *Drug treatment in patients with inflammatory bowel disease. Small italics denote the number of subjects for whom this information was available.*

Statistical analysis

Antibody levels were compared between groups by the Mann-Whitney *U*-test, and numbers of positive results by Fisher's exact test. AAG levels were compared by the two-sample *t*-test (unless otherwise stated in the text). The sign-interval test was used to calculate 95% confidence intervals (CIs) about medians and the *t*-interval test for CIs about means. Correlation was assessed using Pearson's product moment correlation coefficient. The statistical programs *Minitab* and *Arcus* were used to perform the analyses.

RESULTS

Development of ELISAs

Optimal antigen concentration

For each concentration of coating antigen, corresponding pairs of titration curves were compiled from the results obtained with positive and negative sera (Fig. 1 & 2).

At low antigen concentrations, non-specific binding of immunoglobulin in the negative sera was relatively high, presumably due to the availability of binding sites on the plate which were not taken by antigen, and decreased with increasing antigen concentration. However, specific binding of immunoglobulin from the positive sera was seen to diminish quite markedly at high antigen concentrations (especially for sacc) — this latter effect is well-recognised and may be due to steric hindrance from excess antigen bound to the surface of the plate or adsorption of specific immunoglobulin by excess antigen which is loosely bound and therefore more readily eluted during the washing steps.

For the purposes of further experiments, an optimal coating concentration of 50 μg/ml was chosen for sacc, and 0.6 μg/ml for casein.

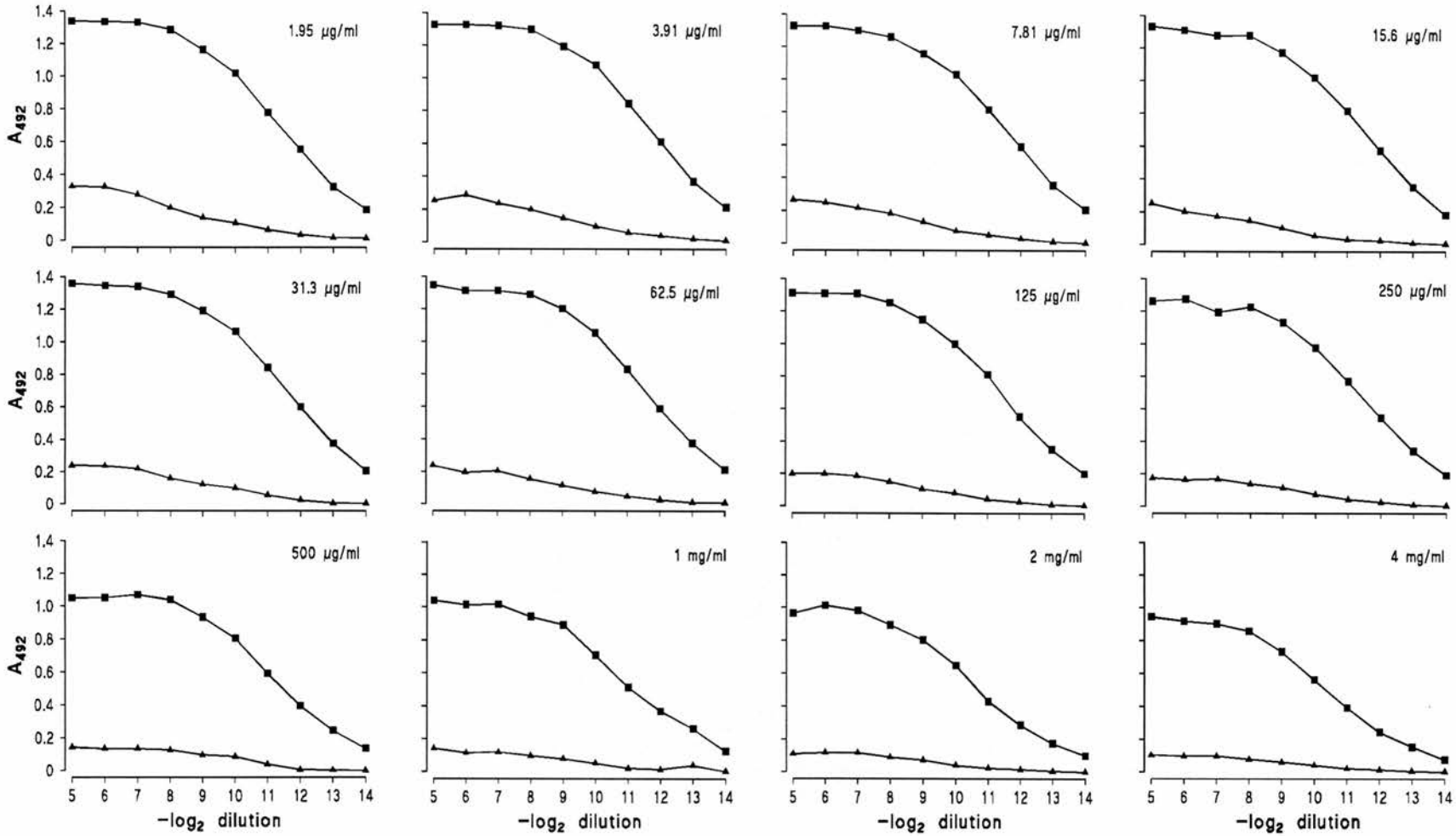

FIGURE 1. *Titration curves obtained with ELISA plates coated with different concentrations of sacc antigen, as shown. Values on the horizontal axis refer to dilutions of normal (triangles) and hyperimmune (squares) rabbit sera.*

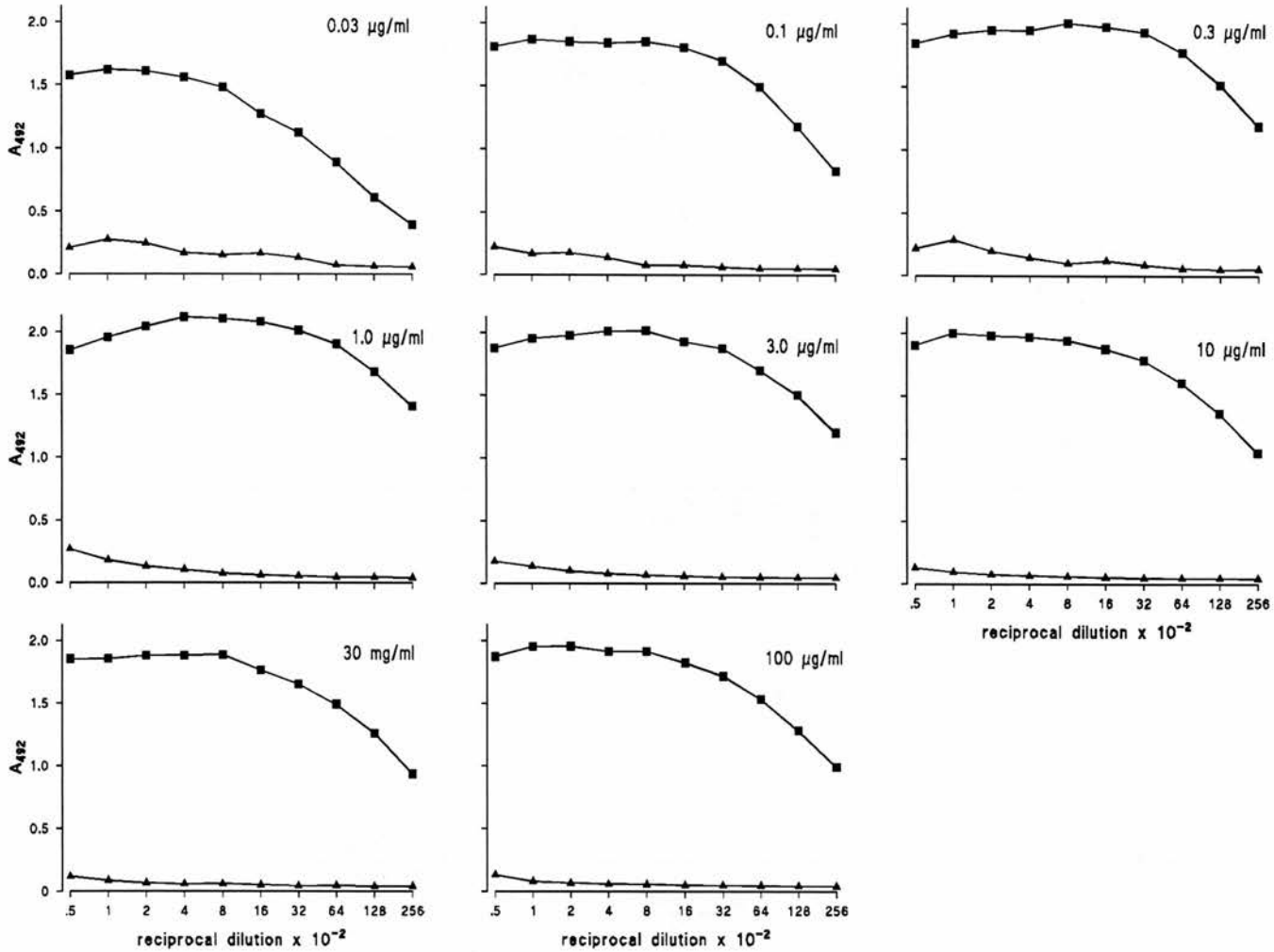

FIGURE 2. *Titration curves obtained with ELISA plates coated with different concentrations of bovine casein, as shown. Values on the horizontal axis refer to dilutions of normal (triangles) and hyperimmune (squares) rabbit sera.*

ASSAY STANDARDS

The absorbance values obtained by screening sera at a single dilution are shown (Fig. 3). Equal volumes of the sera represented by open circles (8 for IgG anti-sacc, 4 for IgA anti-sacc, and 7 for IgG anti-casein) were later pooled to produce calibration standards for each of the specific antibody assays

Representative examples of the calibration curves obtained using the IgG and IgA anti-sacc, and the IgG anti-casein, standards are shown (Fig. 4 & 5).

ASSAY VARIABILITY

Intra-assay variation was very low (<3%) for all three assays (Table 3).

Inter-assay variation was less than 10% for the IgA anti-sacc assay and for the IgG anti-casein assay, but up to 20% for the IgG anti-sacc assay (Table 4). This latter may be an overestimate because the aliquots of serum used had been prediluted prior to freezing — subsequent experience suggests that this may be a factor contributing to poor reproducibility and should be considered technically inadvisable.

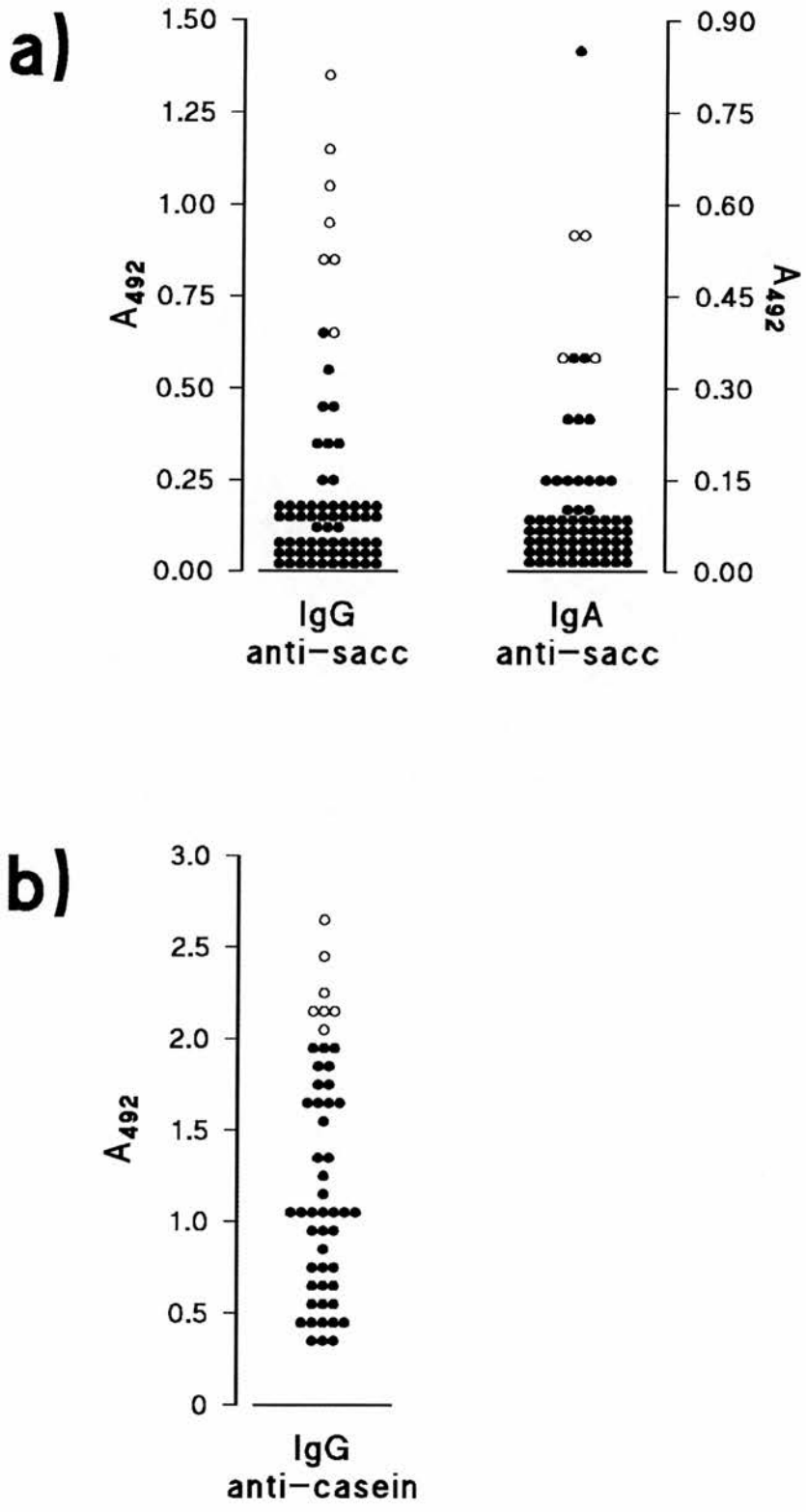

FIGURE 3. *Scatterplot showing absorbance values obtained by screening sera for antibodies to a) sacc, and b) casein. All data have been grouped at intervals of 0.1 absorbance units. Open circles denote sera which were later pooled for use as calibrants.*

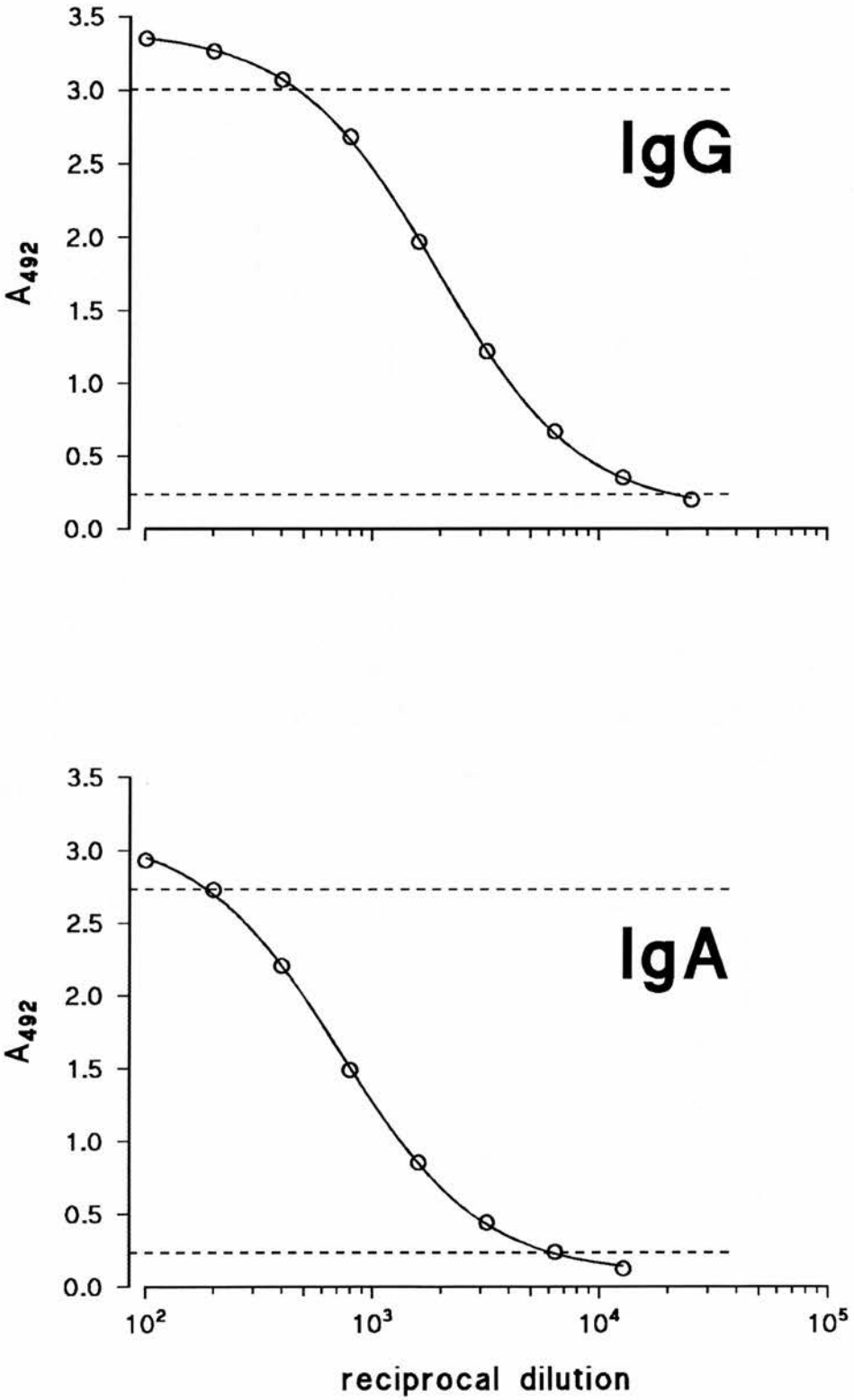

FIGURE 4. *Standard curves obtained with the IgG and IgA anti-sacc calibrants. Dashed lines enclose the part of the curve used to calculate test results.*

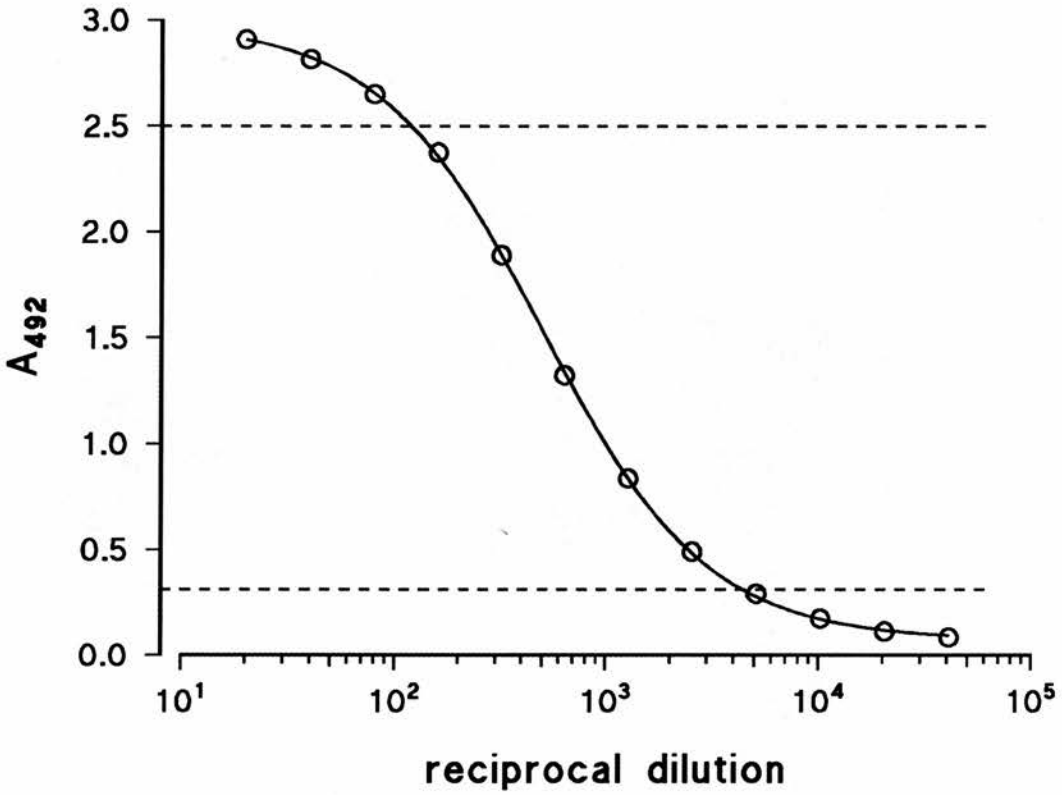

FIGURE 5. *Standard curve obtained with IgG anti-casein calibrant. Dashed lines enclose the part of the curve used to calculate test results.*

Assay	*n*	Relative difference between duplicates (%) (median; 95% C.I.)
IgG anti-sacc	411	2.9; 2.7–3.3
IgA anti-sacc	132	2.1; 1.5–2.9
IgG anti-casein	267	2.8; 2.4–3.6

Table 3. *Intra-assay variation of ELISAs. The number of samples included in each analysis is denoted by* n.

Assay	Sample ID	*n*	Antibody binding (mean; range)		CV (%)
IgG anti-sacc	213	10	1.3;	1.0–1.6	19
	220	18	5.9;	4.6–7.7	15
IgA anti-sacc	220	15	1.8;	1.6–2.1	7.3
IgG anti-casein	105	11	2.5;	2.2–2.8	9.7
	132	12	20.6;	18.7–22.3	6.0
	145	12	32.2;	29.1–35.1	4.7
	82	12	60.4;	51.9–72.2	9.0

Table 4. *Inter-assay variation of ELISAs. The number of times each sample was analysed is denoted by* n.

Anti-sacc antibodies in health and disease

IgG ANTI-SACC

The results of measurement of IgG anti-sacc in the different groups of subjects are shown in Table 5. For the purposes of analysis, sera with IgG anti-sacc below the lowest value which could be quantified were assigned a value of half the limiting value for that particular assay.

Sample distributions were non-normal and were compared using the non-parametric Mann-Whitney test. Both the Crohn's and chronic liver disease groups had IgG anti-sacc levels which were higher than in the normal controls, the differences remaining significant even when corrected for the number of comparisons made.

GROUP	*n*	MEDIAN; 95% C.I.	NO. OF LOW VALUES	*P*
normal	224	2.0; 1.8–2.4	11 (4.9%)	—
Crohn's disease	51	6.6; 3.9–12.2	1 (2.0%)	<0.0001
non-specific colitis	24	1.4; 1.2–2.3	2 (8.3%)	NS
ulcerative colitis	41	2.3; 1.4–3.1	7 (17.1%)	NS
chronic liver disease	23	3.7; 2.1–5.8	0	<0.005
coeliac disease	17	3.0; 2.6–4.0	0	NS
irritable bowel syndrome	7	1.4; 0.7–4.8	1 (14.3%)	NS

TABLE 5. *IgG anti-sacc antibodies. P values are uncorrected for the number of comparisons. 'Low values' denotes sera which were arbitrarily assigned the value of half the lower limit for the assay.*

IgA ANTI-SACC

In the case of IgA anti-sacc, because the proportion of sera with unquantifiably low values was large, use of the Mann-Whitney test was considered inappropriate. Fisher's exact test for 2 × 2 contingency tables was therefore used to

compare groups on the basis of the number of sera with IgA anti-sacc above the estimated 95th. centile for the normal controls, values above this level being considered 'positive'. Again, a significant result was obtained for both the Crohn's and the chronic liver disease group, although, in the latter case, the *P* value was not significant after correction (Table 6).

GROUP	*n*	NO. OF LOW VALUES	NO. "POSITIVE"	*P*
normal	224	180 (80%)	12 (5.4%)	—
Crohn's disease	51	23 (45%)	17 (33%)	3×10^{-7}
non-specific colitis	24	20 (83%)	0	NS
ulcerative colitis	41	25 (61%)	5 (12%)	NS
chronic liver disease	23	12 (52%)	5 (22%)	0.013
coeliac disease	17	12 (71%)	1 (5.9%)	NS
irritable bowel syndrome	7	6 (86%)	0	NS

TABLE 6. *IgA anti-sacc antibodies. P values are uncorrected. Positive values were defined as being greater than the estimated 95th. centile for the normal controls (1.81% of the calibrant). Low values are those which could not be quantified {N.B.* ***not*** *synonymous with negative values}.*

Fig. 6 illustrates graphically the anti-sacc antibody results for the Crohn's and normal groups. Fig. 6b shows how the shape of the distribution of IgA results was affected by the number of unquantifiable values for this isotype (represented by the large blocks of data in the lower tail of the distribution).

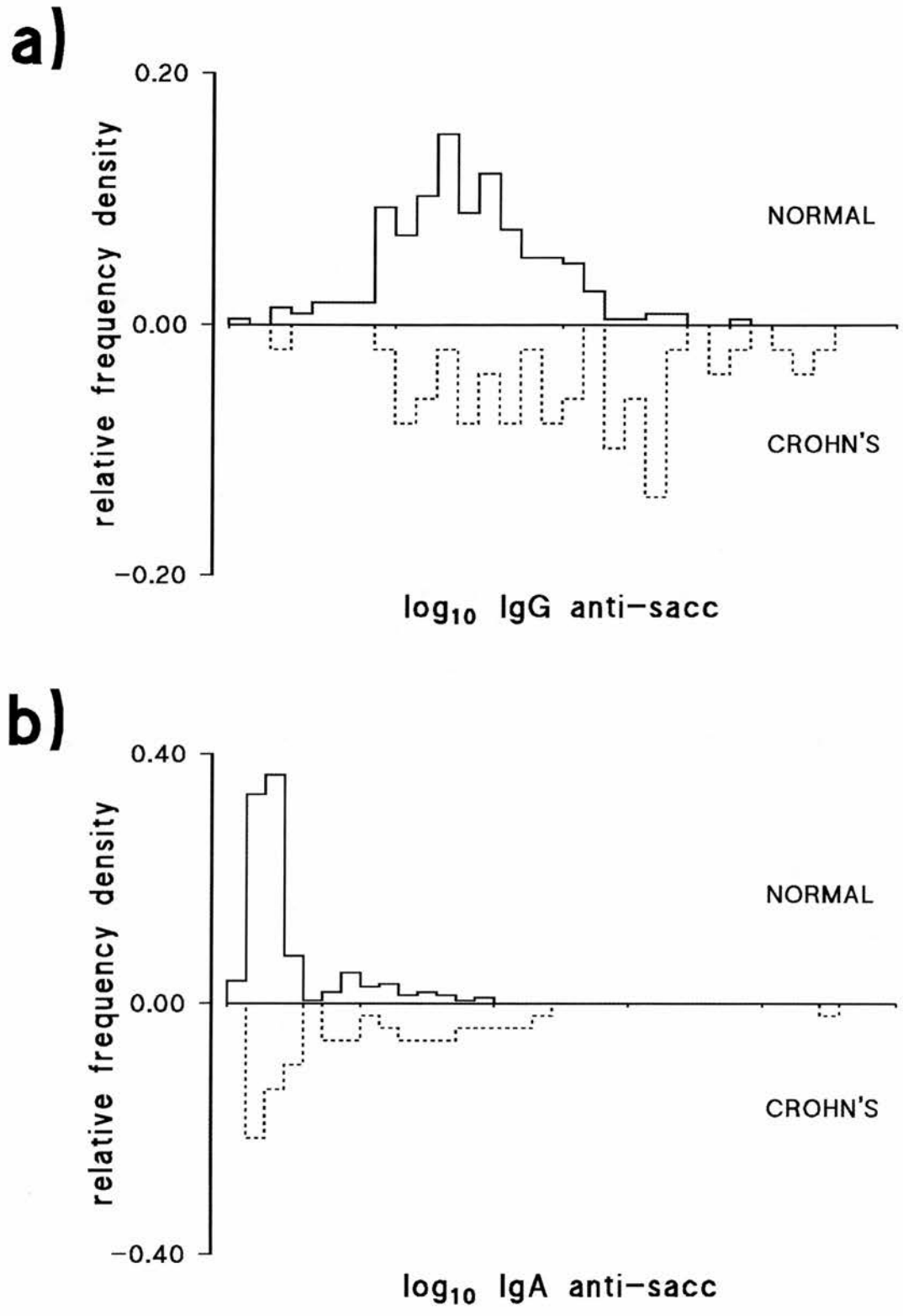

FIGURE 6. *Frequency histograms showing the distributions of a) IgG, and b) IgA isotype-specific antibodies to sacc in normal subjects and Crohn's disease patients. For clarity, the raw data were log-transformed, grouped at intervals of 0.1 and plotted on a linear scale; data from the disease group have been plotted as negative values.*

Further observations in normal subjects

Sex. There was considerable over-representation of males among the normal subjects. However, there was no difference between sexes for IgG anti-sacc (males: 1.96, 1.75–2.43; females: 2.16, 1.70–2.65; $P > 0.94$). In the case of IgA anti-sacc, a higher proportion of positive sera in males also failed to reach statistical significance (males: 11/156 positive; females: 1/68 positive; $P = 0.11$).

Age. There was no correlation between the age of the subjects and the IgG anti-sacc value ($r = 0.067$, $P = 0.34$). Neither was there a correlation between age and IgA anti-sacc after estimating low values in the same way as for IgG ($r = -0.026$, $P = 0.71$); as an additional check, the number of positive results were compared between subjects below the median age of 36 years and those above the median age (4/94 and 6/102, respectively; $P = 0.75$).

Further observations in Crohn's disease patients

Site of disease. A comparison was made within the Crohn's disease group according to whether small bowel disease was present or not. IgG anti-sacc was higher in the presence of small bowel disease, but this observation just failed to reach statistical significance (small ± large bowel disease: 11.4, 4.0–16.2; large bowel disease: 3.8, 2.1–5.5; $P = 0.058$). There was no difference in the proportion positive for IgA anti-sacc (small ± large bowel disease: 12/32 positive; large bowel disease: 3/14 positive; $P = 0.33$). Patients with or without small bowel disease did not differ with respect to severity (AAG 0.95, 0.76–1.15 and 0.95, 0.82–1.37 g/l, respectively; $P = 0.53$, Mann-Whitney. AAG above the upper limit of the normal reference range in 9/32 patients with small bowel disease and in 5/14 of those without small bowel disease;

$P = 0.73$) or duration of disease (78, 46–216 and 61, 21–216 months, respectively; $P = 0.40$, *t*-test).

Duration of disease. Information was available for 46 patients. The median (95% CI) duration was 7 (4.5–13) years. There was no correlation between duration of disease and IgG anti-sacc ($r = 0.24$, $P = 0.11$), but a positive correlation was found between duration and IgA anti-sacc ($r = 0.38$, $P = 0.01$). However there was no difference in the number of positive IgA values between the lower and upper half of the distribution of disease duration (4/23 and 10/23 positive, respectively; $P = 0.11$).

The age was known in 47 patients. There was a highly significant correlation between age and duration of disease ($r = 0.50$, $P = 0.0006$). However, as with normal subjects, there was no correlation between age and IgG ($r = 0.017$, $P = 0.91$) or IgA anti-sacc ($r = -0.015$, $P = 0.92$; 8/23 positive values below the median age and 7/23 above; $P = 1$). Therefore, differences due to age are unlikely confounders in the above analysis. There was no correlation between duration or age and severity of disease ($r = -0.111$, $P = 0.46$ and $r = -0.127$, $P = 0.40$, respectively).

Treatment. Median IgG anti-sacc in those patients taking steroid medication was no different from that in those who were not on this treatment (6.1, 3.5–16.0 and 6.6, 1.8–12.1, respectively; $P = 0.37$). IgA anti-sacc was positive in 7/23 of those on steroids and 7/25 of the others ($P = 1$).

Severity of disease. Serum AAG was obtained for all normal and Crohn's disease subjects; a *Bristol Activity Index* score was available for 38 of the Crohn's disease group. Serum AAG was above the upper limit of the normal range in 16 Crohn's disease patients. It was also higher in this group as a whole, compared with the control subjects (1.10, 0.97–1.23 and 0.74, 0.72–0.76 g/l, respectively; $P < 0.0001$)

and correlated with the *Bristol Activity Index* score ($r = 0.76$, $P = < 0.0001$). Although there was no overall correlation between AAG and IgG anti-sacc ($r = 0.056$, $P = 0.70$), IgG anti-sacc was significantly higher in Crohn's disease patients with an AAG above the normal range, compared with those with normal AAG (13.8, 4.92–19.1 and 5.01, 2.44–10.7, respectively; $P = 0.03$). There was no correlation between AAG and IgA anti-sacc ($r = -0.21$, $P = 0.13$; 5/25 positive in lower half of AAG distribution and 12/25 in upper half; $P = 0.072$); a higher proportion of IgA-positive samples among patients with a high AAG was not statistically significant (8/16 and 9/35, respectively, $P = 0.12$).

IgG ANTI-CASEIN

As a test of whether the above result for the Crohn's disease and chronic liver disease groups could be generalised to another dietary antigen, these sera, and those of the controls, were tested for IgG anti-casein. No significant differences were found between the controls and either disease group (Table 7 and Fig. 7).

GROUP	*n*	MEDIAN; 95% C.I.	NO. OF LOW VALUES
normal	222	7.9; 6.2–10.8	23 (10.4%)
Crohn's disease	51	7.4; 5.2–13.0	3 (5.9%)
chronic liver disease	23	6.9; 4.4–10.5	3 (13.0%)

TABLE 7. *IgG anti-casein antibodies.*

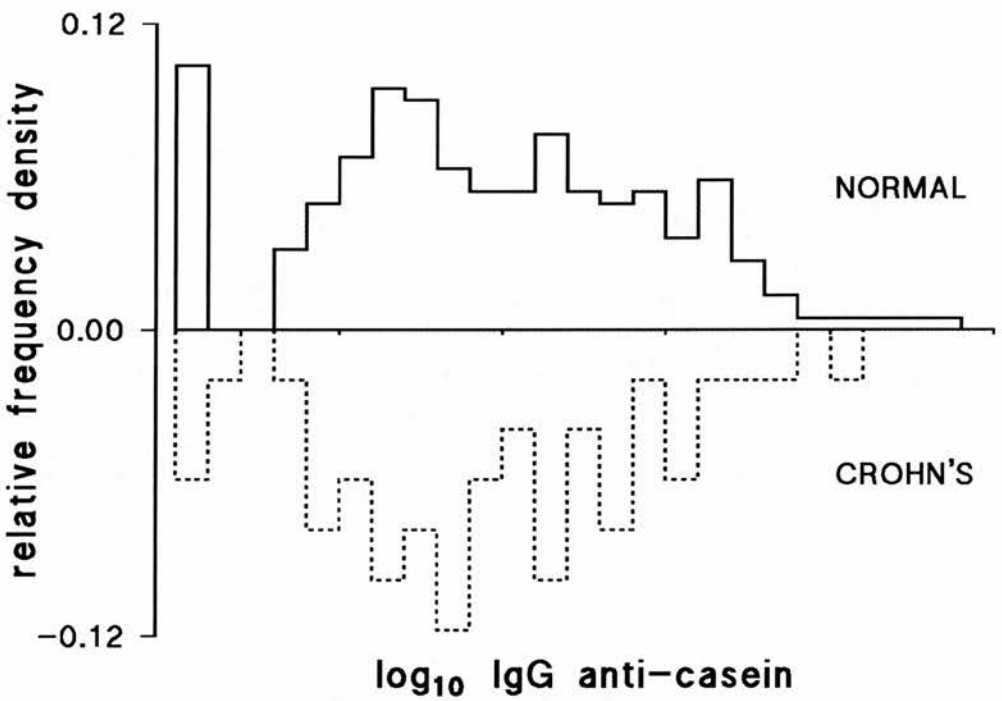

FIGURE 7. *Frequency histogram showing the distribution of IgG anti-casein antibodies in normal subjects and Crohn's disease patients. Data have been treated as for the previous figure.*

II

Observations on cellular responses to sacc

MATERIALS AND METHODS

Preparation of cells

PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC)

PBMC were separated over ficoll by the method of Böyum.[210] Over time, minor empirical adjustments to the technique were made, and the final form is described here.

Aliquots of whole blood were collected from donors by venepuncture and diluted with an equal volume of sterile PBS containing preservative-free heparin to give a final concentration of 15 u/ml. This mixture was carefully decanted onto a *Lymphoprep (Nycomed)* density gradient and centrifuged at 600 × *g* (1,637 rpm[a]) for 20 min. at 20 °C. The mononuclear cell layer at the plasma/ficoll interface was removed by pipette and the cells washed once in an excess of sterile PBS, followed by centrifugation at 400 × *g* (1,337 rpm) for 15 min at 20 °C. The supernatant was discarded and the cells were resuspended in a small volume of complete culture medium. After adding a small aliquot of the suspension to an equal volume of trypan

a: speeds are quoted for the Mistral 3000i centrifuge

blue dye, a manual count of viable cells was obtained using a haemocytometer counting chamber (modified Neuebauer), and corrected for the dilution factor. Cells obtained by this method were virtually 100% viable.

UMBILICAL CORD BLOOD MONONUCLEAR CELLS (CBMC)

Fresh umbilical cord blood was obtained from placentae as soon as possible after delivery, with the permission of the mothers. Cells prepared from umbilical cord blood by the above method were heavily contaminated with red cells. Therefore, heparinised cord blood was first depleted of erythrocytes by adding 2 ml 1% w/v methylcellulose *(Sigma)* in PBS to each 10 ml aliquot, mixing for 15 min. at 37 °C, then diluting with an equal volume of PBS and allowing erythrocytes to sediment for 30 min. The leucocyte-rich supernatant was then subjected to density gradient centrifugation as described for PBMC.

SHEEP ERYTHROCYTE ROSETTES

Preparation of sheep red blood cells (SRBC). Sheep blood was collected into an equal volume of Alsever's solution and washed three times with sterile PBS, each time centrifuging at 400 × *g* for 15 min. at 20 °C and removing any buffy coat. Packed cells were pipetted into 2-aminoethylisothiouronium bromide hydrobromide solution[241] (AET; see Appendix I) *(Sigma)*, incubated for 30 min. at 37 °C and washed three times as before. The erythrocytes were then resuspended at 4% v/v in sterile PBS and stored at 4 °C until required.

Rosetting procedure. Rosetting was performed on PBMC obtained as described above. Using 2.5 ml of the 4% SRBC suspension for each 10 ml of the original blood

sample from which the PBMC were obtained, the erythrocytes were washed once more, as described above, just before use. The PBMC suspension was added and the mixture centrifuged gently at 223 × *g* (1,000 rpm) for 5 min. at 20 °C to enhance rosette formation. The cells were then resuspended and separated over *Lymphoprep*.

Non-rosetting (E^-) cells. Buoyant, non-rosetting mononuclear cells were aspirated from the top of the density gradient and washed once with sterile PBS, centrifuging at 322 × *g* (1,200 rpm) for 12.5 min. at 20 °C. Erythrocyte lysing buffer (see Appendix I) was added to the resuspended cells, and, after mixing for 1 min., an excess of sterile PBS was added. Following centrifugation at 322 × *g* for 10 min. at 20 °C, the E^- cells were resuspended in complete culture medium before counting. This preparation was used as a source of antigen-presenting cells (APC).

Rosetting (E^+) cells. After removal of E^- cells, the supernatant and density gradient were aspirated and discarded. The rosetted cells were then resuspended and washed once with sterile PBS, centrifuging at 322 × *g* for 10 min. at 20 °C. The cells were again resuspended and the density gradient separation step repeated. After a further wash, the rosettes were treated with lysing buffer, washed and counted, as for E^- cells. The E^+ cells thus obtained were used as a source of T-lymphocytes.

REMOVAL OF ADHERENT CELLS

For some experiments, E^+ cells were further depleted of antigen-presenting cells by adherence of the latter to plastic surfaces. Cells were incubated for 1 hour at 37 °C, either in a small volume (~5 ml) in a sterile Petri dish or in a loosely packed nylon wool column, in the barrel of a 10 ml syringe. In each case, non-adherent cells were eluted with RPMI before washing and resuspending in complete

culture medium.

$CD4^+$ AND $CD8^+$ T-CELLS

T-lymphocytes expressing the CD4 or CD8 co-receptor molecules were obtained by sequential positive selection using paramagnetic beads bearing covalently linked surface mouse IgM monoclonal antibodies directed against specific epitopes on the target molecules.

E^+ cells obtained from 80 ml blood, and 250 µl *Dynabead-M450-CD4 (Dynal 111.15)* were washed separately with sterile PBS containing 5% v/v fetal calf serum (PBS/FCS). After discarding the supernatants, the beads were resuspended in ~200 µl PBS/FCS and added to the cell pellet, with thorough mixing. Following incubation for 30 min. at room temperature, with intermittent mixing, 10 ml PBS/FCS were added and the tube was placed in the magnetic separator for 2 min. The supernatant, containing unbound cells, was poured off and subjected to two further depletions of magnetic particles to remove all CD4 beads. Similarly, the beads, with their bound cells, were washed twice to remove contaminating unbound cells and then resuspended in a small volume (~50 µl) of PBS/FCS. Cells were released from the *Dynabeads* by adding 20µl *DETACHaBEAD (Dynal 125.03)*, containing goat or sheep poyclonal antibodies specific for mouse Fab epitopes, and incubating for 30 min. at room temperature, with intermittent mixing. Finally, the beads were removed from the detached cells by means of three depletions with the magnet, following which the cells were centrifuged and resuspended in complete culture medium.

The original supernatant fraction from the above was then processed in an

identical manner with *Dynabead-M450-CD8 (Dynal 111.07)*.

CD45RO$^+$ AND CD45RA$^+$ T-CELLS

Subpopulations of T-lymphocytes expressing the RO and RA isotypes of the CD45 molecule were prepared by negative selection of CD4$^+$ cells, obtained as described above, using *Dynabeads* in an indirect method.

Two aliquots of CD4$^+$ cells, each containing ~20 × 10^6 cells, were centrifuged at 322 × *g* for 6 min. and the supernatant removed. The cells were then incubated for 30 min. at 4 °C in the presence of 20 µl of mouse anti-human CD45RO or mouse anti-human CD45RA *(Dako)* monoclonal antibody. After three washes in PBS/FCS, to remove excess antibody, 250 µl of *Dynabeads (Dynal 110.05)*, coated with a primary goat antibody specific for the Fc region of mouse immunoglobulin, were added to each tube, followed by a further incubation for 30 min. at 4 °C. Unbound cells were then subjected to three depletions of magnetic particles and bound cells, washed and suspended in complete culture medium.

Flow cytometric analysis of cell populations

Surface phenotyping of cells was performed on a *Coulter XL* flow cytometer equipped for three-colour analysis. Data were saved in listmode files and analysed using *WinMDI* version 2.1.4.

When staining cells, ~2 × 10^5 cells were used for each antibody or combination of antibodies. Cells were washed once with PBS/FCS and centrifuged at 322 × *g* for 6 min. After removing the supernatant, 5 µl of the relevant antibody preparation(s) were added and mixed well with the cells, which were then incubated

for 30 min. at room temperature. Following a further wash, the cells were resuspended in 1 ml PBS/FCS, and 1 ml 2% w/v paraformaldehyde in PBS was then added. Tubes were kept at 4 °C until analysed.

All staining was *direct*, and the antibody-fluorochrome conjugates used are listed in Table 8.

ANTIBODY CLONE & ISOTYPE		SPECIFICITY	FLUOROCHROME	SOURCE
TÜK4	$IgG_{2a}\kappa$	CD14	FITC	*Dako* F0844
HD37	$IgG_1\kappa$	CD19	FITC	*Dako* F0768
UCHT1	$IgG_1\kappa$	CD3	RPE	*Dako* R0810
UCHT1	$IgG_1\kappa$	CD3	RPE-Cy5	*Dako* C7067
UCHT1 MT310	$IgG_1\kappa$ $IgG_1\kappa$	CD3 CD4	FITC RPE	*Dako* FR875
UCHT1 DK25	$IgG_1\kappa$ $IgG_1\kappa$	CD3 CD8	FITC RPE	*Dako* FR881
HIT3a SFCI12T4D11 SFCI21Thy2D3	IgG_{2a} IgG_1 IgG_1	CD3 CD4 CD8	ECD RD1 (RPE) FITC	*Coulter* 6604654
UCHL1	$IgG_{2a}\kappa$	CD45RO	FITC	*Dako* F0800
4KB5	$IgG_1\kappa$	CD45RA	RPE	*Dako* R7086

Table 8. *Antibody-fluorochrome conjugates used to label cells for flow cytometry.*

Cell culture

Culture medium

All cultures were maintained in 'complete culture medium'. RPMI 1640 medium *(Flow or Gibco)* containing 25 mM HEPES buffer was purchased without L-glutamine or bicarbonate. To each 100 ml were added 3 ml 7.5% w/v $NaHCO_3$ solution *(Sigma or Gibco)*; 12 ml heat-inactivated (56 °C for 30 min.), sterile-filtered (0.22 μm), human AB serum; 2 ml L-glutamine 200 mM *(Gibco)* and 2 ml of a solution containing penicillin 5,000 iu/ml plus streptomycin 5,000 μg/ml *(Gibco)*.

Antigens and mitogens

Sacc. An aqueous extract of dried baker's yeast was prepared as previously described.

Purified protein derivative of Mycobacterium tuberculosis (PPD). This was obtained as a pharmaceutical preparation containing 100,000 u/ml tuberculin *(Evans Medical)*.

Tetanus toxoid (TT). Unadsorbed tetanus vaccine, 60 ml *(Evans Medical)* was dialysed extensively against distilled water, sterile filtered (0.22 μm) and lyophilised. It was then redissolved in 10 ml distilled water, centrifuged and re-filtered.

Streptokinase/streptodornase (SK). 20 ml of distilled water were added to a vial of *Varidase Topical (Lederle Laboratories)*, according to the manufacturer's instructions.

Pokeweed mitogen (PWM). A stock solution of 2 mg/ml was prepared by dissolving 10 mg lyophilised lectin from *Phytolacca americana (Sigma)* in 5 ml sterile water.

Phytohaemagglutinin (PHA). A stock solution of 400 μg/ml was prepared by dissolving 2 mg lyophilised lectin from *Phaseolus vulgaris (Murex)* in 5 ml sterile water.

Interleukin-2 (IL-2). A stock solution of 10^4 u/ml was prepared as a 1/10 dilution of purified lymphoblastoid IL-2 *(Biotest)* in RPMI 1640. Preliminary experiments were conducted to assess the activity of this preparation (see Appendix II).

All of the above were stored at −20 to −30 °C until required.

PROLIFERATION ASSAYS

For experiments using PBMC or CBMC as responder cells, cells were adjusted to 2/3 × 10^6/ml in culture medium and deposited into sterile 96-well, U-bottomed culture plates *(Costar or Flow)* at 10^5 cells in 150 µl/well. Dilutions of antigens/mitogens were prepared in culture medium at four times their intended final concentrations in culture and added to the wells in 50 µl volumes; the same volume of culture medium alone was added to control wells. The number of replicate wells varied between experiments. Plates were then placed in a 37 °C incubator with a humidified atmosphere containing 5% CO_2.

On the day of harvesting, the cells were pulsed with 1 µCi of [methyl-^{3}H]-thymidine ([^{3}H]-Tdr) *(Amersham)* by adding 1 µl of a 1 mCi/ml solution to each well and incubating for a further six hours. The cells were then harvested on to glass fibre filters using a semi-automatic cell harvester *(Flow)*. The filters were allowed to dry overnight prior to β-counting of the individual discs in 2 ml *OptiScint 'Hi Safe'* liquid scintillant *(LKB)*, using a 2 minute counting protocol. For some experiments, a *Topcount* system *(Hewlett-Packard)* was used, which allowed whole plates to be harvested in a single manoeuvre and counted in the 96-well format.

Median counts per minute (c.p.m.) were calculated for each group of replicate wells. Results were expressed either as absolute c.p.m. or as the stimulation index:

$$\text{S.I.} = \frac{\text{median c.p.m. for stimulated cultures}}{\text{median c.p.m. for unstimulated cultures}}$$

For proliferation assays in which the responder cells were separated T-cells, lines or clones, the responder cells were incubated in the presence of irradiated PBMC or E^- cells. A preliminary experiment was conducted to assess the efficacy of irradiation in preventing thymidine uptake (see Appendix III).

T-CELL LINES AND CLONES

PHA-stimulated clones. PBMC obtained from a single donor were cloned by limiting dilution as follows: viable cells were cultured at 0.1 lymphocyte/well in the presence of PHA 1 μg/ml, IL-2 50 u/ml and irradiated PBMC at 5×10^4/well in 96-well culture plates. After 14 days, wells which demonstrated growth were expanded into flasks containing 15 ml culture medium, IL-2 50 u/ml and irradiated cells at 3×10^5/ml. The medium was replaced after a further seven days, and the cells were harvested three days later (*i.e.*, ten days after the last addition of feeder cells).

Sacc-specific lines and clones. PBMC from the same donor as above were cultured at 10^5/well in the presence of sacc at 1/10 the concentration which produced maximal stimulation (this resulted in approximately half-maximal [^{3}H]-Tdr uptake when assessed at 7 days). On day 5, IL-2 was added at 10 u/ml. Thereafter, the cells were expanded into culture tubes and then flasks, with repeated rounds of stimulation with sacc, IL-2 and irradiated syngeneic E^- cells.

From this cell line, after approximately six rounds of stimulation, cloning was performed by limiting dilution: responder cells were cultured at 1, 3 and 10/well in the presence of sacc, IL-2 and irradiated E^- cells at 2×10^4/well. Wells which

showed growth were then expanded as above.

LIMITING DILUTION ANALYSIS OF PRECURSOR CELL FREQUENCY

A variable number of E^+ responder cells was cultured in the presence of 2×10^4 irradiated E^- cells in multiple replicate wells. At each responder cell input, half of the replicate wells were unstimulated and half stimulated with the antigen under investigation. After six days, the cultures were pulsed with [^{3}H]-Tdr, harvested and β-counted. Stimulated wells giving a count greater than the mean + 3 SD of the counts from the corresponding unstimulated wells were considered to have shown a positive response.

Results were analysed using the GLIM program *(Royal Statistical Society)*, which calculates precursor frequency by a maximum likelihood method, and deviation from single-hit kinetics by a χ^2 test of goodness-of-fit.

CELL-MEDIATED CYTOTOXICITY ASSAY

Effector cells. PBMC were cultured in bulk at 5×10^5 lymphocytes/ml in 50 ml flasks. Four cultures were established for each donor, as follows: unstimulated cells, cells plus sacc, cells plus PPD and cells plus IL-2. After seven days, the cells were washed in RPMI supplemented with 10% FCS, resuspended in a small volume of medium, and a viable count performed. Finally, the cells were adjusted to 5×10^5/ml.

Target cells. Two cell lines were used: mel-1, an NK-resistant line derived from a malignant melanoma;[242] and molt-4, an NK-sensitive line of T-cell leukaemia origin[243] *(these were kindly provided by Dr. Steve Christmas)*. These were harvested from culture, washed twice in RPMI/10% FCS and resuspended in the presence of 10 μl

of a 10 mCi/ml solution of ^{51}Cr-sodium chromate *(Flow)*. After incubation for one hour at 37 °C, the cells were washed twice in RPMI/10% FCS and adjusted to 5×10^4/ml after performing a viability count.

The assay was performed in triplicate, by adding 150 µl of each effector cell suspension to the same volume of each target cell suspension in 11 mm diameter LP3 tubes, to give an effector to target cell ratio of 10:1. After gentle centrifugation at 1,000 rpm ($223 \times g$) for 5 min., followed by incubation for four hours at 37 °C, 150 µl of supernatant were carefully removed from each tube without disturbing the cell pellet. Respective pairs of pellets and supernatants were then counted in a γ-counter. Fractional release for each pair of tubes was calculated as:

$$R = \frac{\text{c.p.m. of supernatant}}{\text{c.p.m. of supernatant + c.p.m. of pellet}}$$

Spontaneous release was calculated from tubes containing target cells plus 150 µl medium only, and maximum release from tubes containing target cells plus 150 µl 2% Triton 100 *(BDH)* to lyse the cells. A mean background count was subtracted from each experimental count, and the % cytotoxicity for each pair of tubes calculated as:

$$\% \text{ cytotoxicity} = \frac{R_{test} - R_{spont}}{R_{max} - R_{spont}} \times 100\%$$

where:

R_{test} = fractional release in test sample

R_{spont} = mean spontaneous fractional release

R_{max} = mean maximum fractional release

The mean of each triplicate was taken as the final result.

Sacc-specific cellular response in Crohn's disease

SUBJECTS

Blood was collected from 20 patients with Crohn's disease and 26 laboratory staff, who acted as controls.

Of the Crohn's disease patients, 6 were male and 14 female. The median age was 40 years (range: 18–66 years) and the median (95% CI) disease duration was 11 years (5.2–16.8 years). Small bowel disease (with or without large bowel disease) was present in 14, and 6 had large bowel disease only. Steroids were the sole treatment in 2 patients, 5-ASA derivatives in 6, and 4 patients were taking both medications. None of these patients had been included in the antibody study described in Part I.

Of the control subjects, 11 were male and 15 female. The median age was 36 years (range: 24–49 years).

PBMC were separated as described and serum was stored. Proliferation assays were carried out with PBMC from each subject to assess the response to sacc, SK, TT and PPD. Cultures, in replicates of six, were harvested at 6 days. Serum IgG anti-sacc was measured in all subjects, and AAG in the Crohn's disease group.

RESULTS

Response of PBMC to sacc

DOSE-DEPENDENCY

The [^{3}H]-Tdr uptake, after seven days in culture, of PBMC from normal donors in response to four-fold dilutions of sacc, PPD and PWM is shown in Fig. 8 & 9. No subject failed to respond to sacc or PPD, although it can be seen that the response varied considerably between individuals, especially when the stimulus was sub-maximal. Even when only the maximum SIs achieved by each subject were considered (*i.e.* regardless of dose), the response was still highly variable (Table 9).

An optimal response to sacc appears to be more critically dependent on dose than is the case for PPD and PWM, which both produce a shallow plateau of responses over a greater than 200-fold concentration range (Fig. 9). However, this observation is probably an artefact consequent on excessive dilution of the culture medium when larger amounts of the sacc solution were added.

STIMULUS	MEDIAN S.I.	95% C.I.	RANGE
sacc	84	47.4–203.1	7.8–617
PPD	154.5	75.8–315.4	7.9–1007

TABLE 9. *Maximum stimulation indices in response to sacc and PPD in healthy subjects.*

TIME COURSE

Data on the time-course of the proliferative responses were obtained by measuring [^{3}H]-Tdr incorporation daily in the presence of the concentration of sacc,

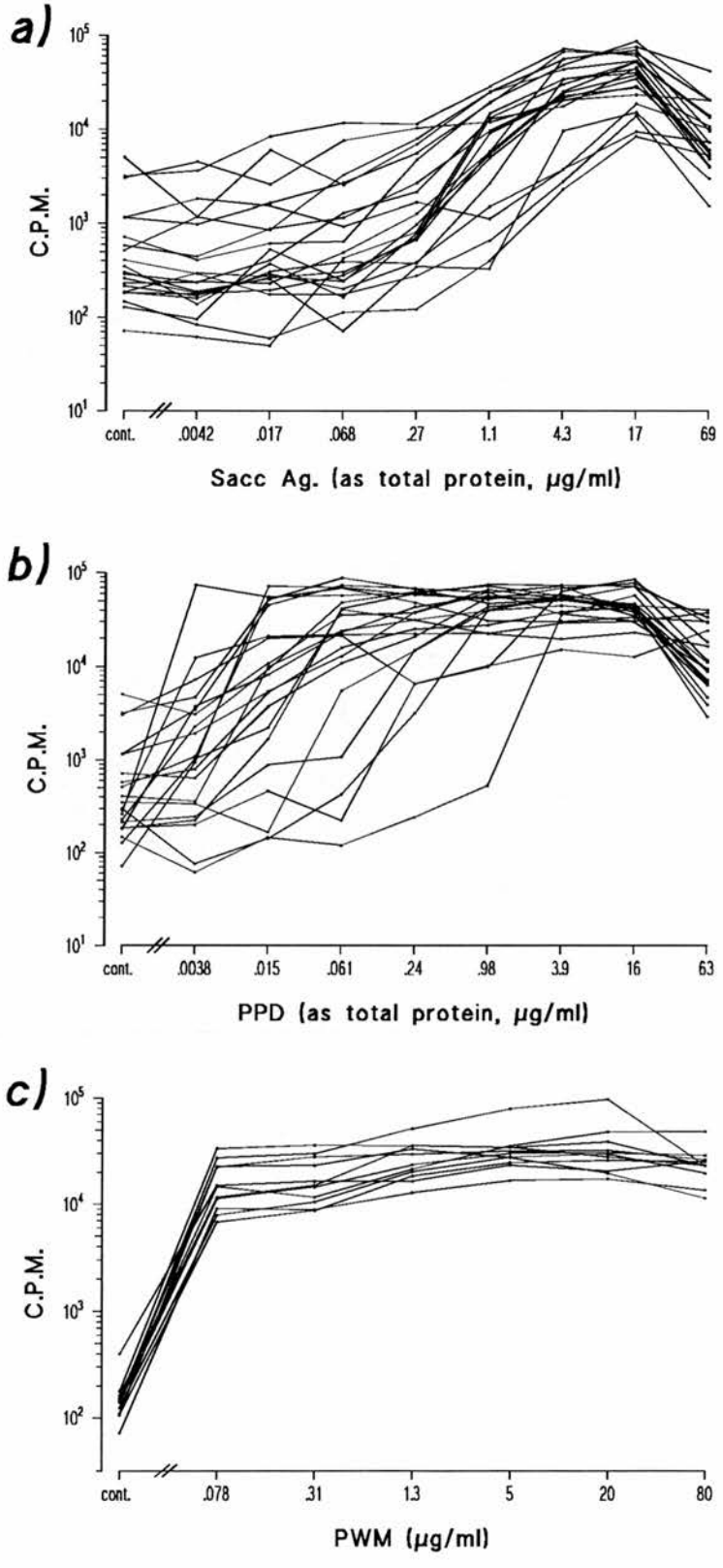

FIGURE 8. *Dose-response of PBMC from normal subjects to sacc, PPD and PWM. Individual data are shown, where each point represents the median of eight replicate counts taken after seven days in culture.*

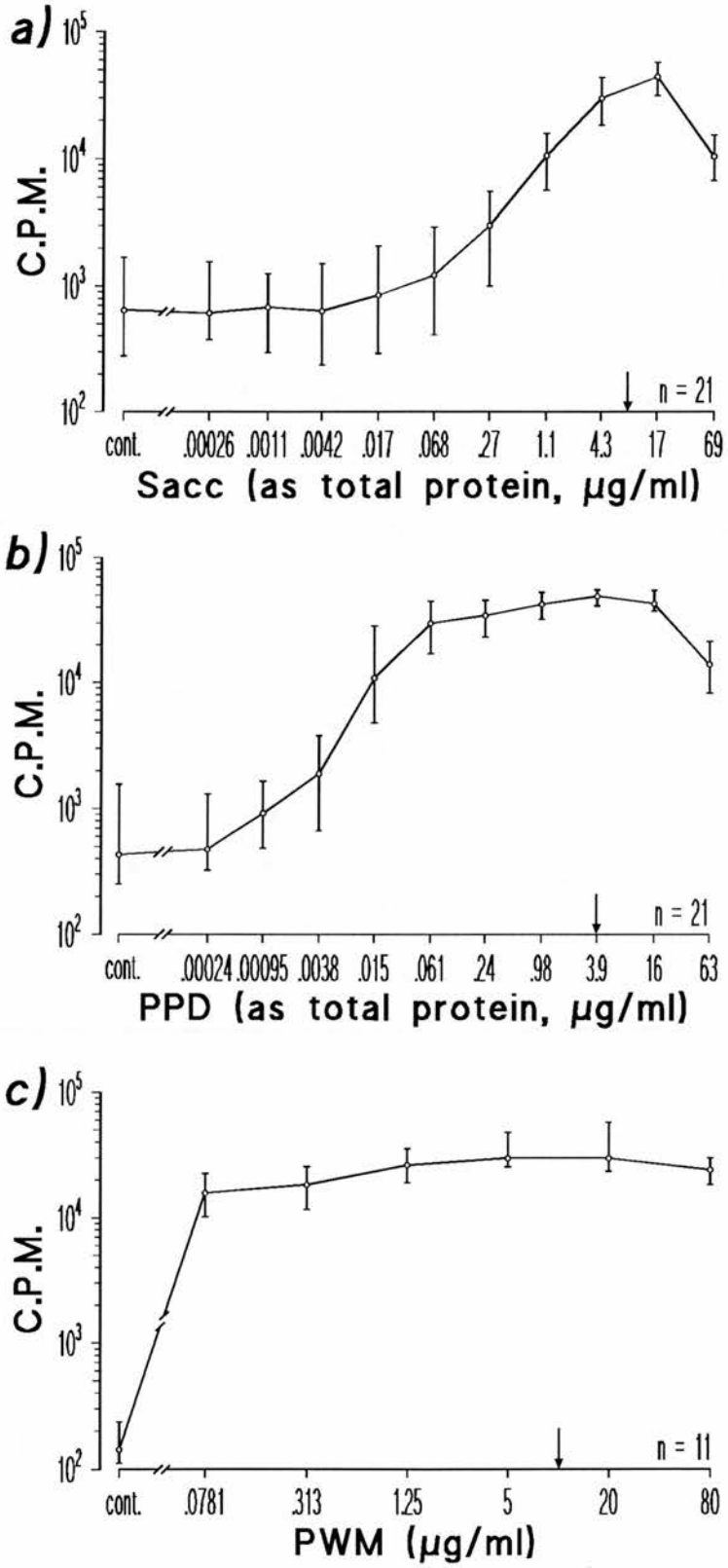

FIGURE 9. *Dose-response of PBMC from normal subjects to sacc, PPD and PWM. Data shown in Fig. 8 have been condensed to medians and 95% confidence intervals for the sample. Arrows show concentrations used to assess time course of the response.*

PPD and PWM which had produced the optimal response at seven days. Whereas PWM elicited very early proliferation which was maintained throughout the course of the experiment, peak responses to sacc and PPD were delayed at 7 days and 5-6 days respectively (Fig. 10).

Response of CBMC to sacc

CBMC were incubated with concentrations of sacc, PPD, TT, PHA[a] and PWM which had been shown to be optimal for adult PBMC. The incubation period was 4 days for PHA and 7 days for the other stimuli. At the end of this time, the incorporation of [^{3}H]-Tdr was compared with that of similar cultures of normal adult PBMC.

When expressed as absolute counts per minute, the background response of CBMC cultures was significantly higher, and the PPD response significantly lower, than that of PBMC (Fig. 11a). Although all stimulation indices were significantly lower for CBMC (Fig. 11b), this was probably a consequence of the markedly elevated background uptake.

a: the dose-response profile for PHA was assessed separately and is not shown

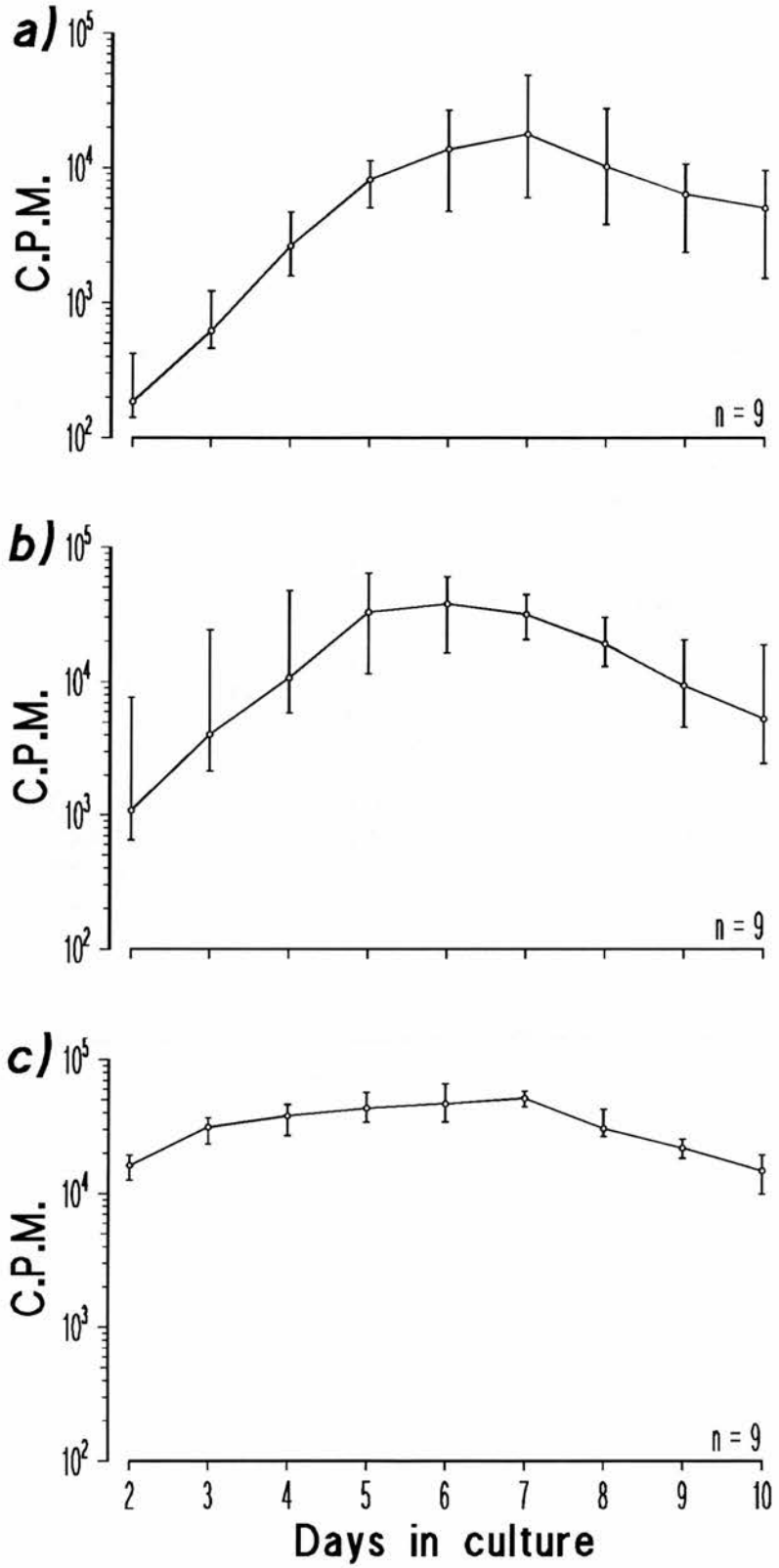

FIGURE 10. *Time course of PBMC response to a) sacc, b) PPD, and c) PWM. Medians and 95% confidence intervals are shown; these were compiled from individual responses, which were medians of six replicates.*

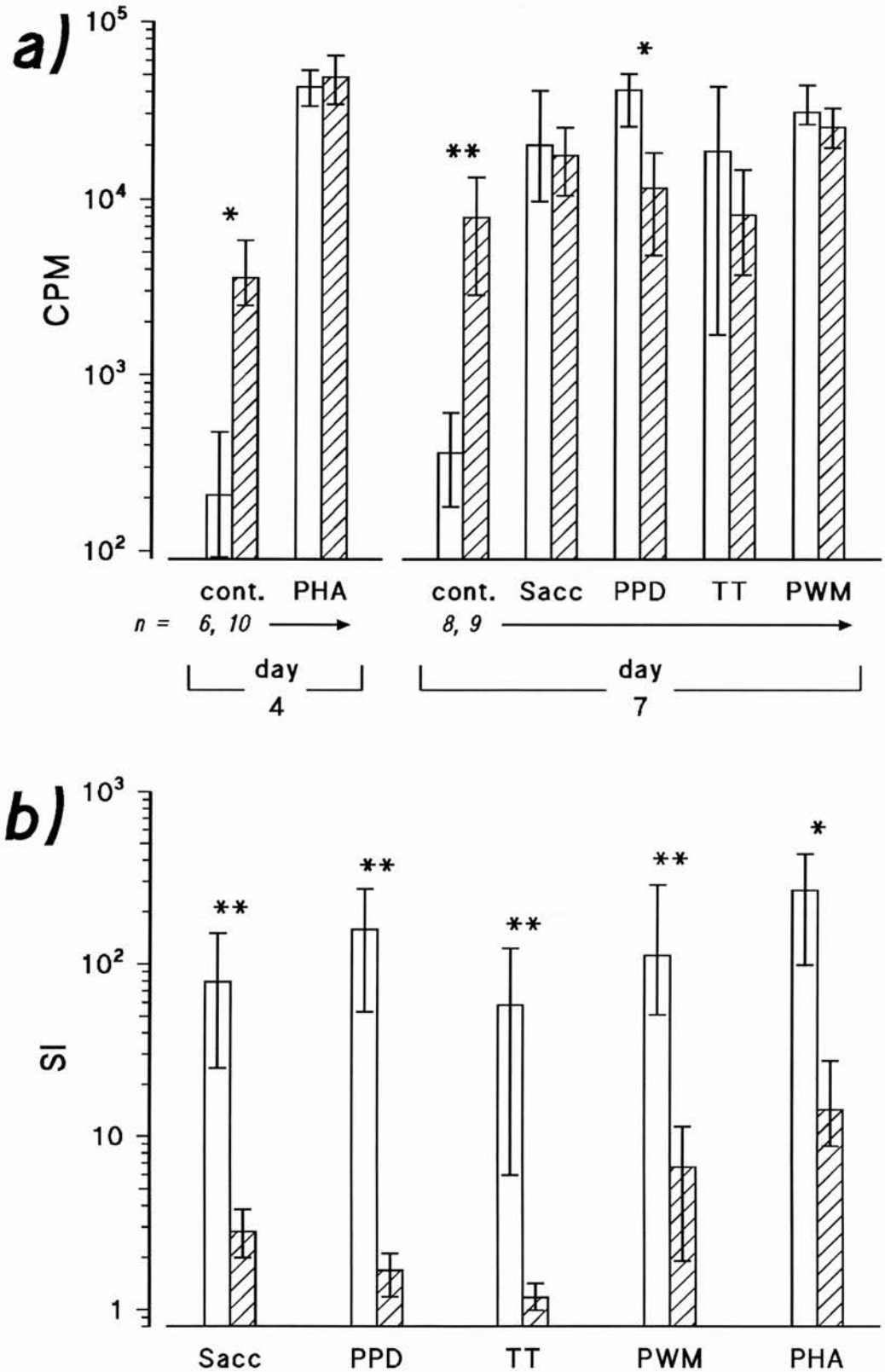

FIGURE 11. *Response of CBMC (shaded bars) and PBMC (open bars) to sacc, recall antigens and mitogens. a) counts per minute; b) stimulation indices. Error bars represent 95% confidence intervals about the median. * = $P < 0.05$; ** = $P < 0.01$ (Mann-Whitney).*

Responses of separated cell populations

The responses of separated cells were examined in a healthy subject whose PBMC had responded to sacc with a SI of >100.

ROSETTING AND NON-ROSETTING CELLS

Effectiveness of rosetting. The effectiveness of sheep cell rosetting in separating T-cells from PBMC is demonstrated in Fig. 12, 13 and Table 10. $CD3^+$ T-cells were depleted in the non-resetting (E^-) population and enriched in the rosetting (E^+) population, with reciprocal changes in $CD14^+$ (monocyte/macrophage) cells and $CD19^+$ B-cells.

MARKER	PBMC	E^-	E^+	MORPHOLOGICAL GATE
CD3	72.2 (67.6)	5.6 (2.5)	86.5 (82.7)	lymphocyte (all cells)
CD19	7.5 (6.0)	33.3 (14.9)	0.05 (0.05)	lymphocyte (all cells)
CD14	0.43 (5.02)	16.7 (40.4)	<0.1	all cells (monocyte)

TABLE 10. *Cell surface markers before and after rosetting. All figures are per cent.*

T-cells as responder cells: dependency on APCs. When cells were separated by rosetting and selectively irradiated, responsiveness to sacc, SK and TT was seen only when both T-cells and APC were present in culture. The response was abrogated by irradiation of the T-cells but was maintained if the APC were irradiated. The response to PHA was less sensitive to depletion of APCs (Fig. 14).

Supplementation of T-cells with increasing numbers of APCs had a dose-dependent effect on the restoration of responsiveness to sacc (Fig. 15).

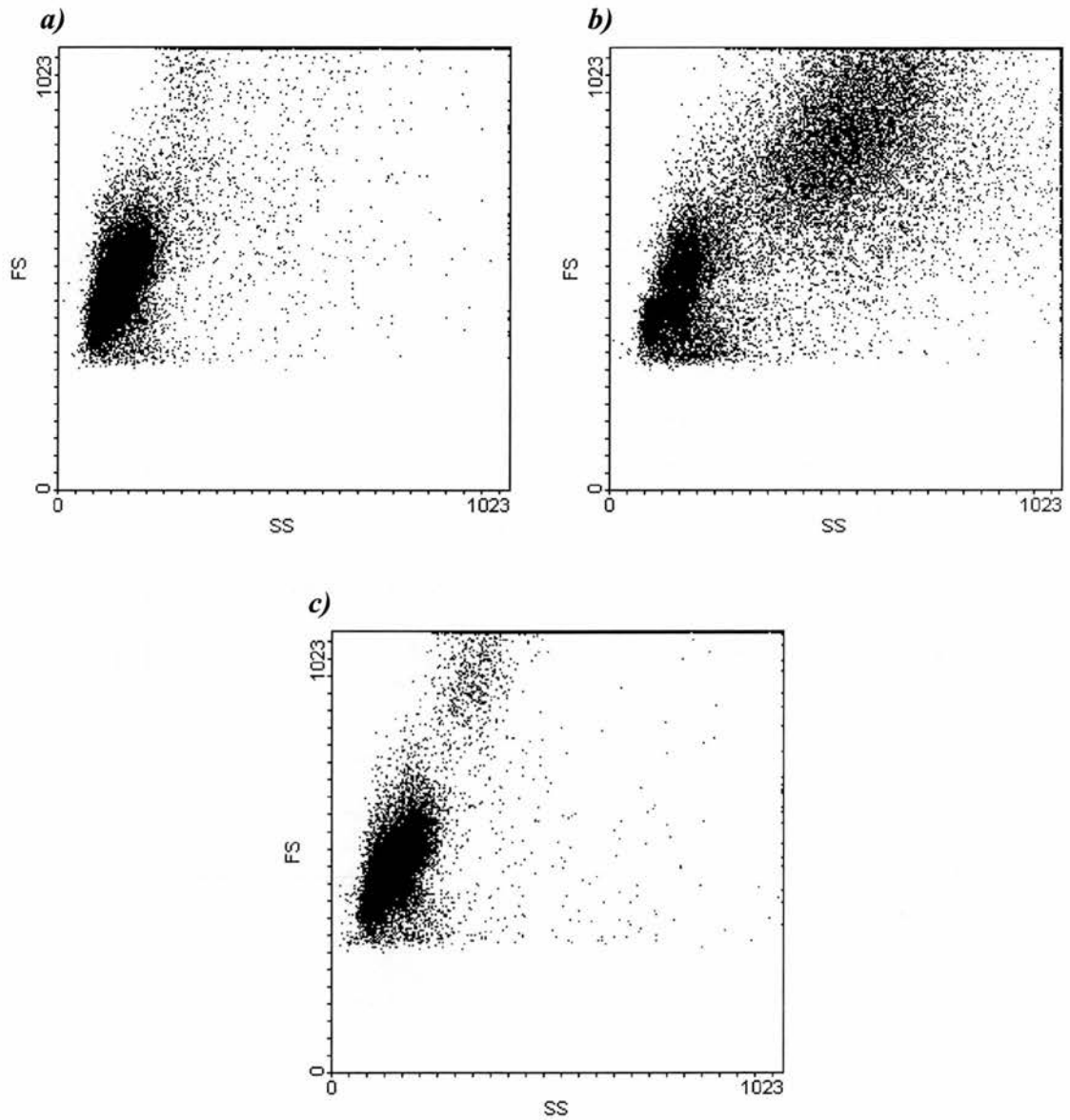

FIGURE 12. *Morphology of cells, by flow cytometry, before and after separation by sheep erythrocyte rosetting: a) PBMC, b) E^-, c) E^+. In each panel, the main population, at lower left, is of lymphocytes. The enrichment of E^- cells with monocytes is clearly shown in 'b' as a large population of cells with higher FS and SS. (FS = forward scatter, SS = side scatter.)*

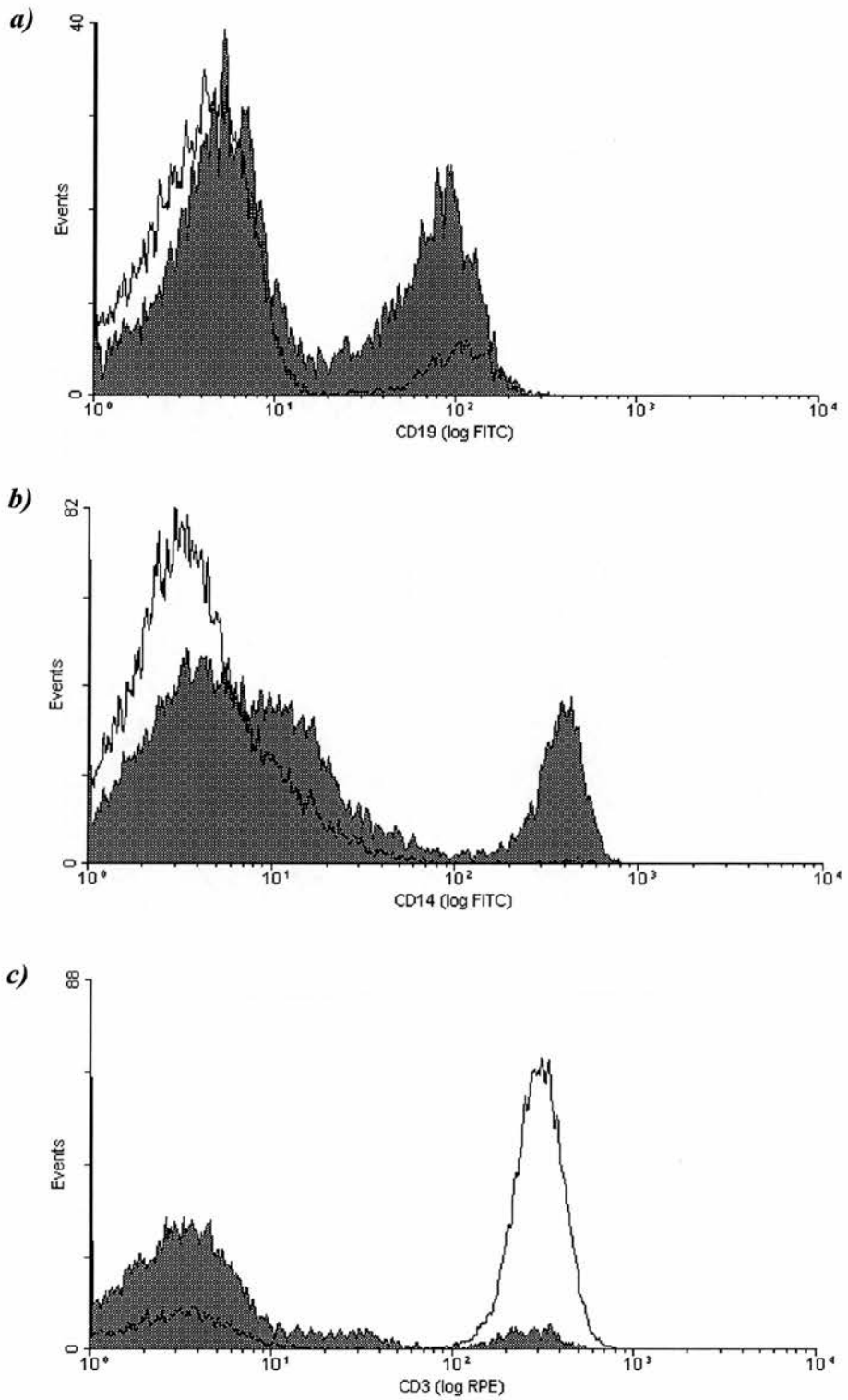

FIGURE 13. *Cell surface markers before and after rosetting. Shaded histograms are: a) CD19 on E^- cells, b) CD14 on E^- cells, c) CD3 on E^- cells, d) CD3 on E^+ cells, and e) CD19 on E^+ cells. Outline histograms represent the same analysis on PBMC, and are normalised for numbers of events.*

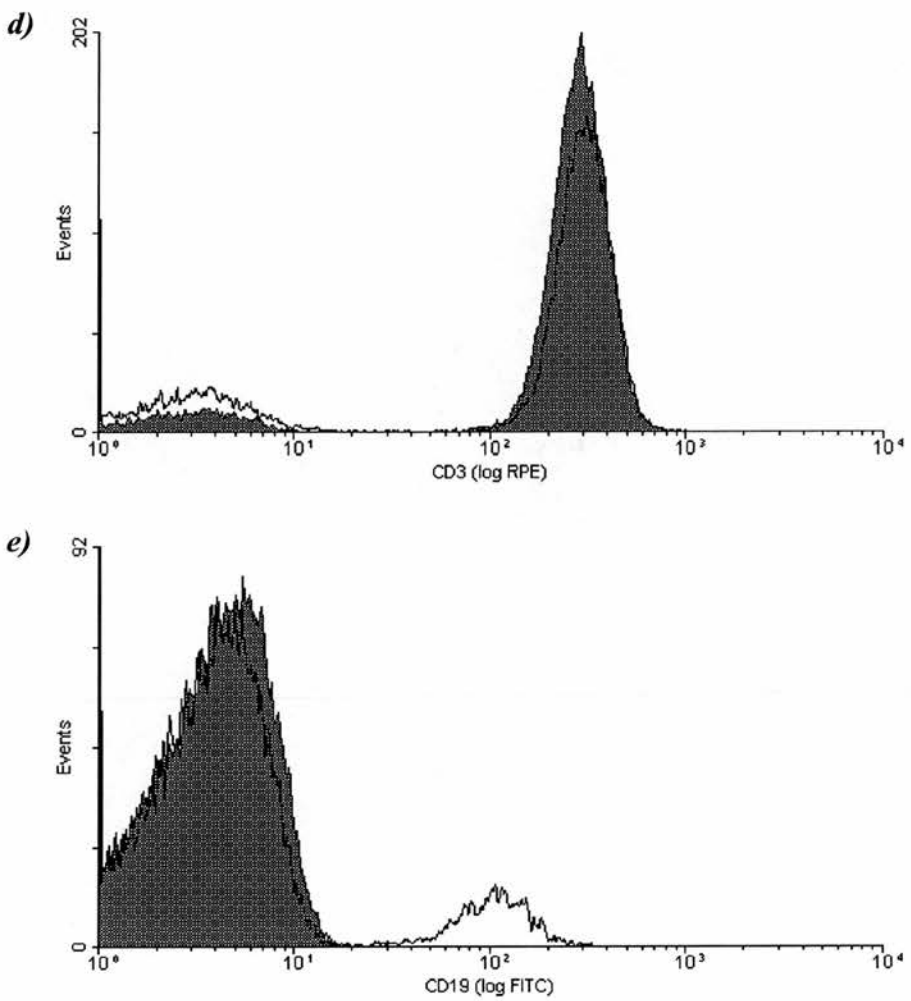

FIGURE 13. *cont.*

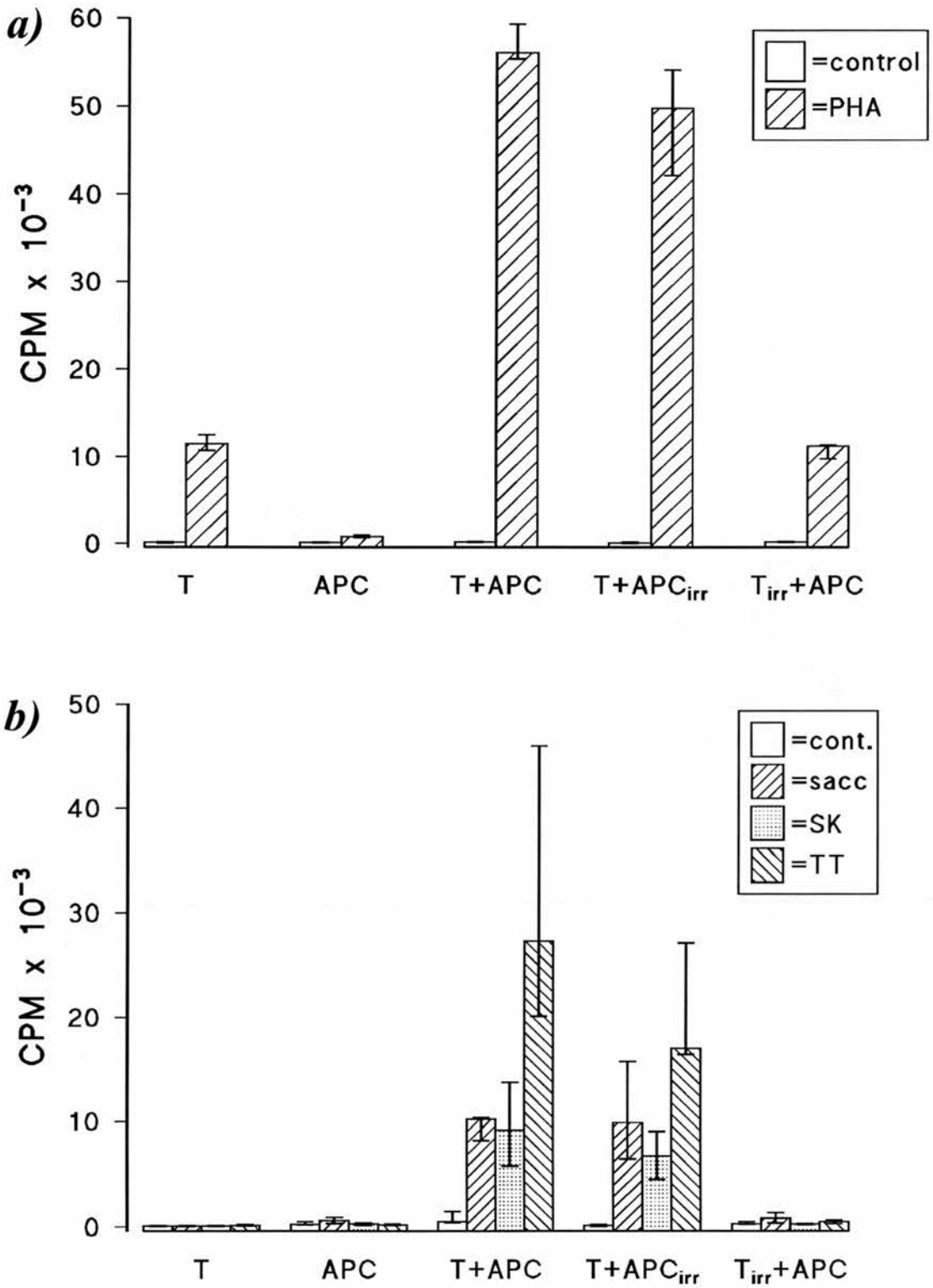

FIGURE 14. *Response of separated cells to a) PHA, b) sacc, SK and TT. Bars represent medians and ranges of five replicates. (T = E^+ cells at 10^5/well, APC = E^- cells at 2 × 10^4/well; E^+ cells were depleted of contaminating adherent cells.)*

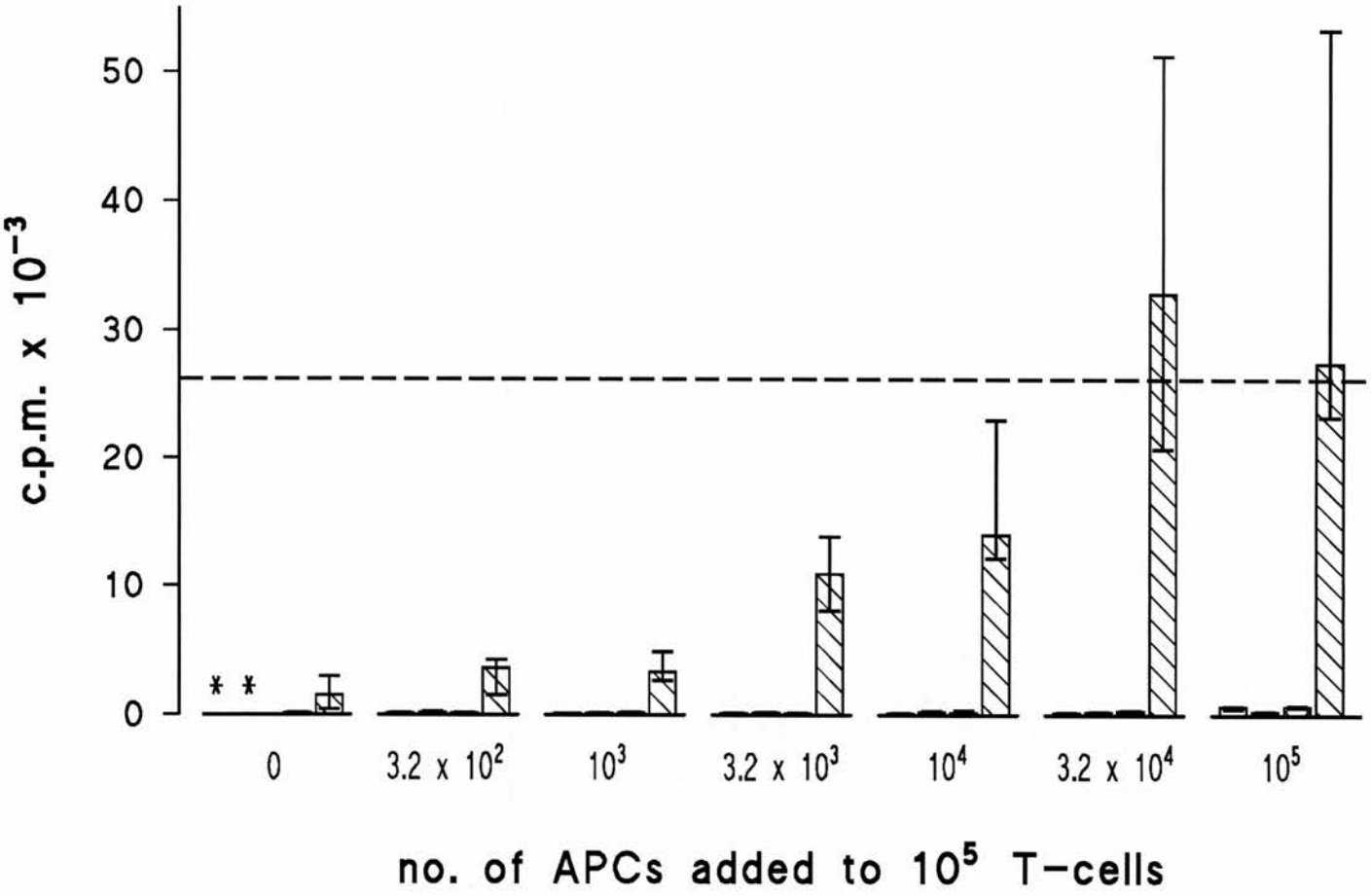

FIGURE 15. *Dependency of sacc response on APC. Bars represent the median and range of five replicates. There are four counts for each input of APCs: APCs unstimulated, APCs stimulated, APCs + T-cells unstimulated, APCs + T-cells stimulated (from left to right). The dashed line represents the median count obtained with 10^5 unseparated PBMC. T-cells were depleted of contaminating adherent cells.*

$CD4^+$ AND $CD8^+$ CELLS

A preliminary experiment was done, using PBMC from three healthy donors, to compare the phenotypic characteristics of cells after stimulation with sacc, SK, TT and PHA, with those of control cells, incubated with culture medium only. In all cases, sacc, SK and TT lead to a relative rise in the proportion of cells expressing CD4 and a reciprocal fall in $CD8^+$ cells. The change was particularly marked among cells with high forward and side scatter (as defined by the gating region R_2). By contrast, in PHA-stimulated cells, any rise in CD4 relative to CD8 expression was much less marked, and, in one case, this ratio fell (Fig. 16 & 17).

PBMC from one of the above donors were then separated into $CD4^+$ and $CD8^+$ populations, prior to stimulation in the presence of irradiated APC. The separation was highly efficient, with >97% purity being obtained in each case (Fig. 18). Only $CD4^+$ responded to sacc, SK and TT, whereas Both $CD4^+$ and $CD8^+$ cells responded to PHA and PWM (Fig. 19 and Table 11).

$CD45RO^+$ AND $CD45RA^+$ CELLS

$CD4^+$ cells from the above donor were further separated into cells expressing CD45RO and CD45RA. In each case, >93% purity was achieved (Fig. 20). The response of $CD45RO^+$ cells to sacc, SK and TT was considerably greater than that of $CD45RA^+$ cells. By contrast, the response to PWM was greater for the $CD45RA^+$ population (Fig. 21 and Table 12).

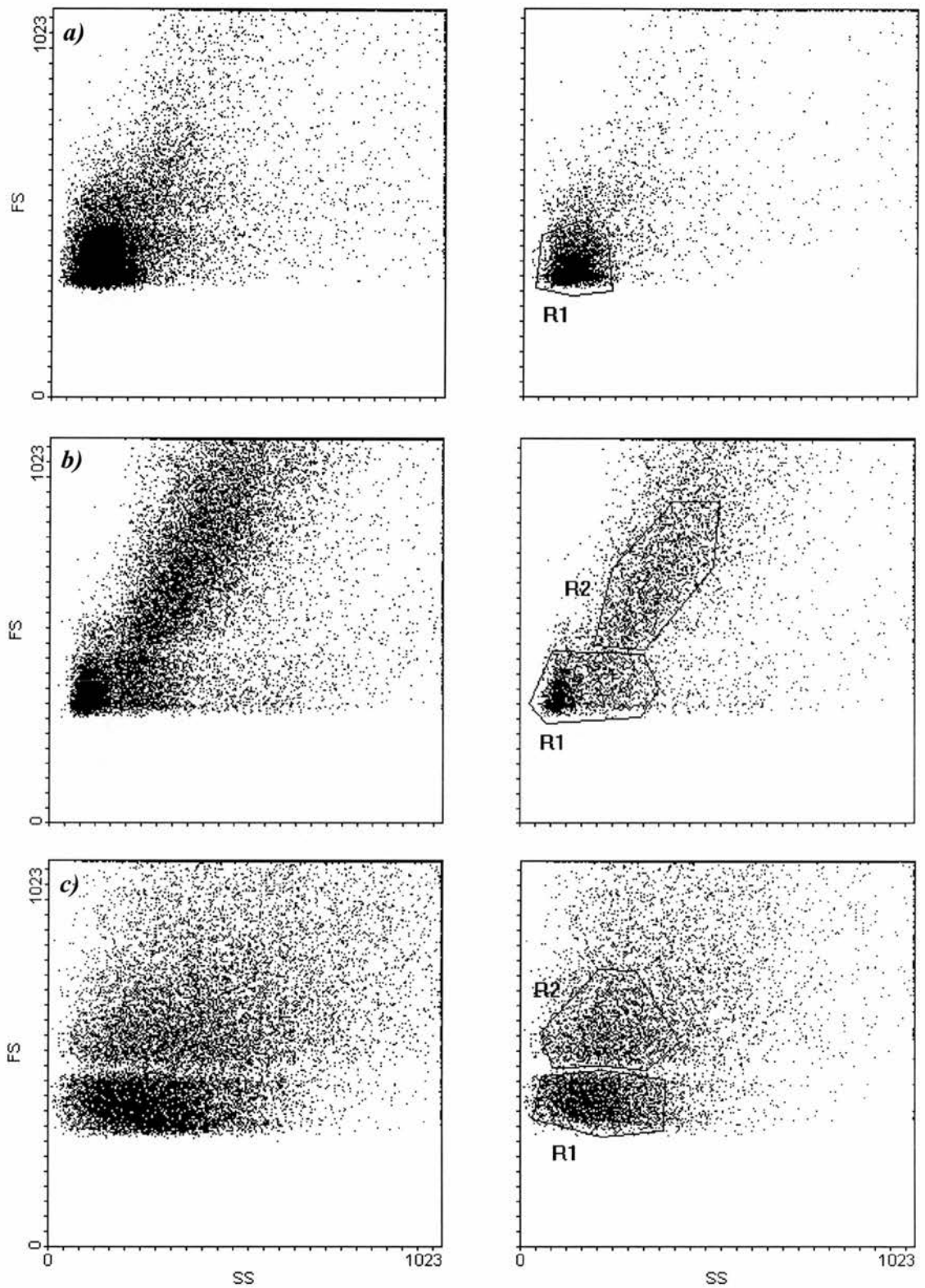

FIGURE 16. *Gating of cells after stimulation. Panels on right are as those on left but showing fewer events, and with gates superimposed: a) control, b) sacc (the appearance was similar for SK and TT), c) PHA. Cells in gates R1 and R2 were analysed for expression of CD3, CD4 and CD8.*

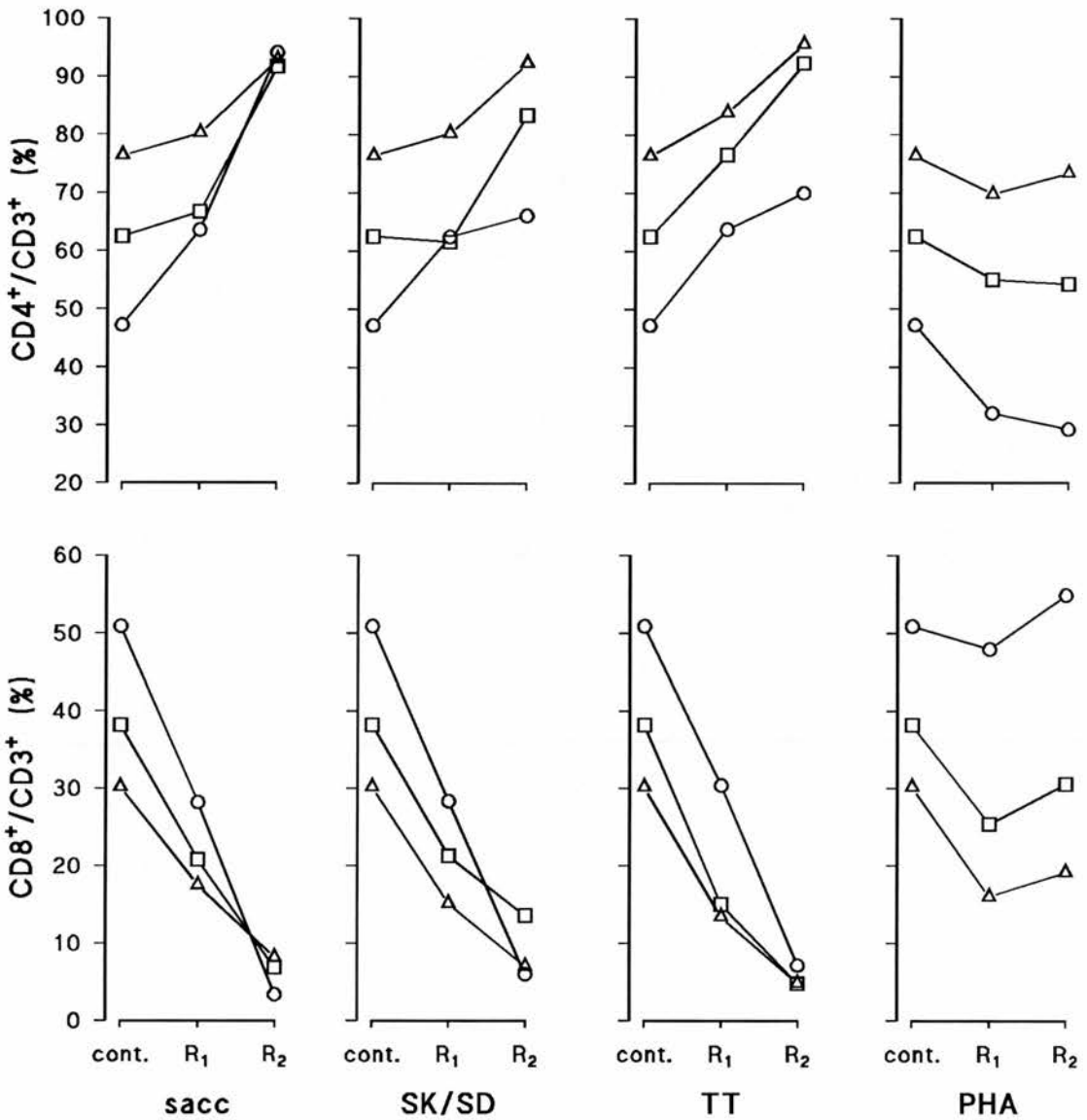

FIGURE 17. *Surface markers of cells after stimulation. R1 and R2 are morphological gates as shown in Fig. 16. Within these, only $CD3^+$ were examined further for CD4 and CD8 expression. Symbols represent three different donors.*

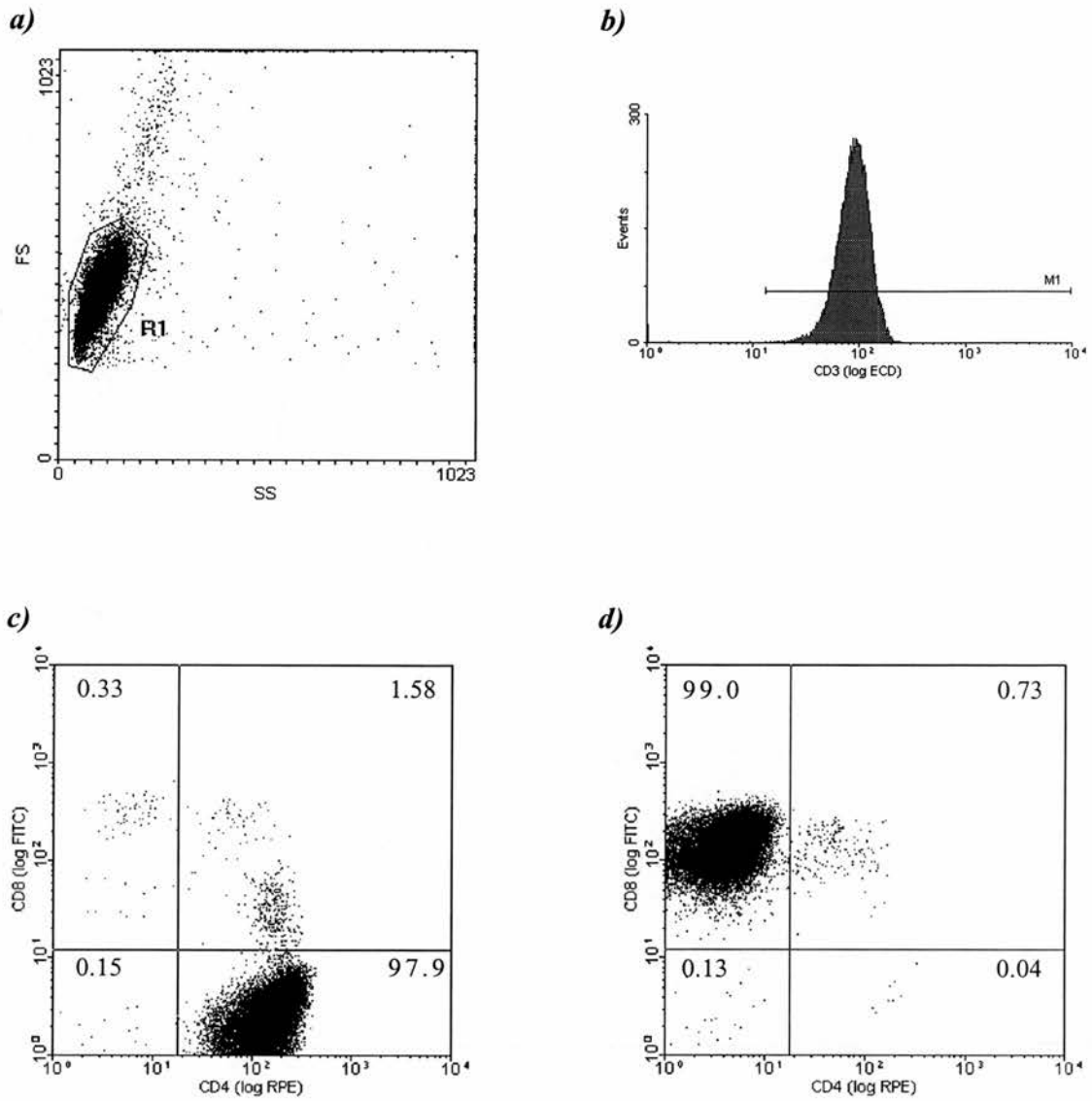

FIGURE 18. *Flow cytometric analysis of separated CD4⁺ and CD8⁺ T-cells: a) dotplot showing FS/SS of cells positively selected on CD4 — region R1 was used as a lymphocyte gate, b) histogram showing CD3 expression of cells in R1 — marker M1 was used to define a further logical gate for CD3⁺ lymphocytes, c) dotplot showing CD4 and CD8 expression of cells gated on R1 and M1 — numbers in each quadrant represent percentages of total events within the gate, d) dotplot analogous to that in 'c' but obtained with cells positively selected on CD8.*

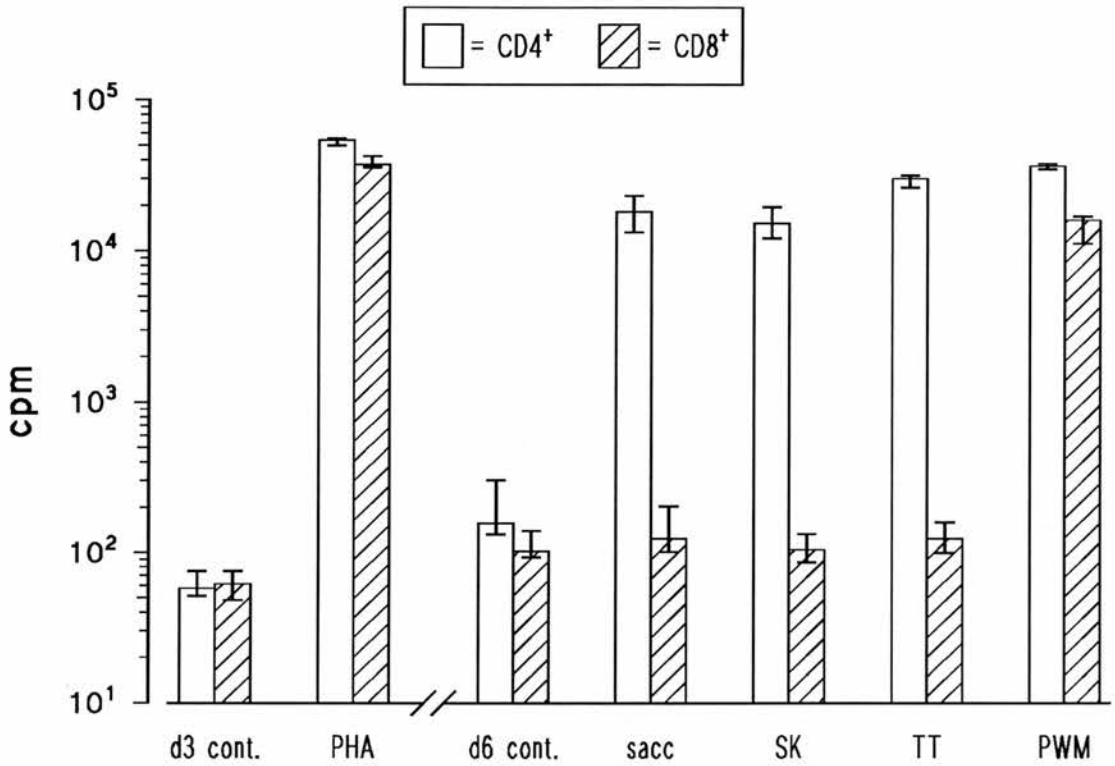

FIGURE 19. *Response of $CD4^+$ and $CD8^+$ T-cells to sacc, mitogens and recall antigens. Bars represent the median and range of five replicate counts.*

STIMULUS	$CD4^+$		$CD8^+$	
day 3 control	58	—	62	—
PHA	53,863	(929)	37,168	(599)
day 6 control	157	—	102	—
sacc	18,099	(115)	124	(1.2)
SK/SD	15,166	(97)	104	(1.02)
TT	30,228	(193)	124	(1.2)
PWM	36,754	(234)	16,061	(157)

TABLE 11. *Data from Fig. 19. Numbers are median counts per minute of five replicates, with stimulation indices in brackets.*

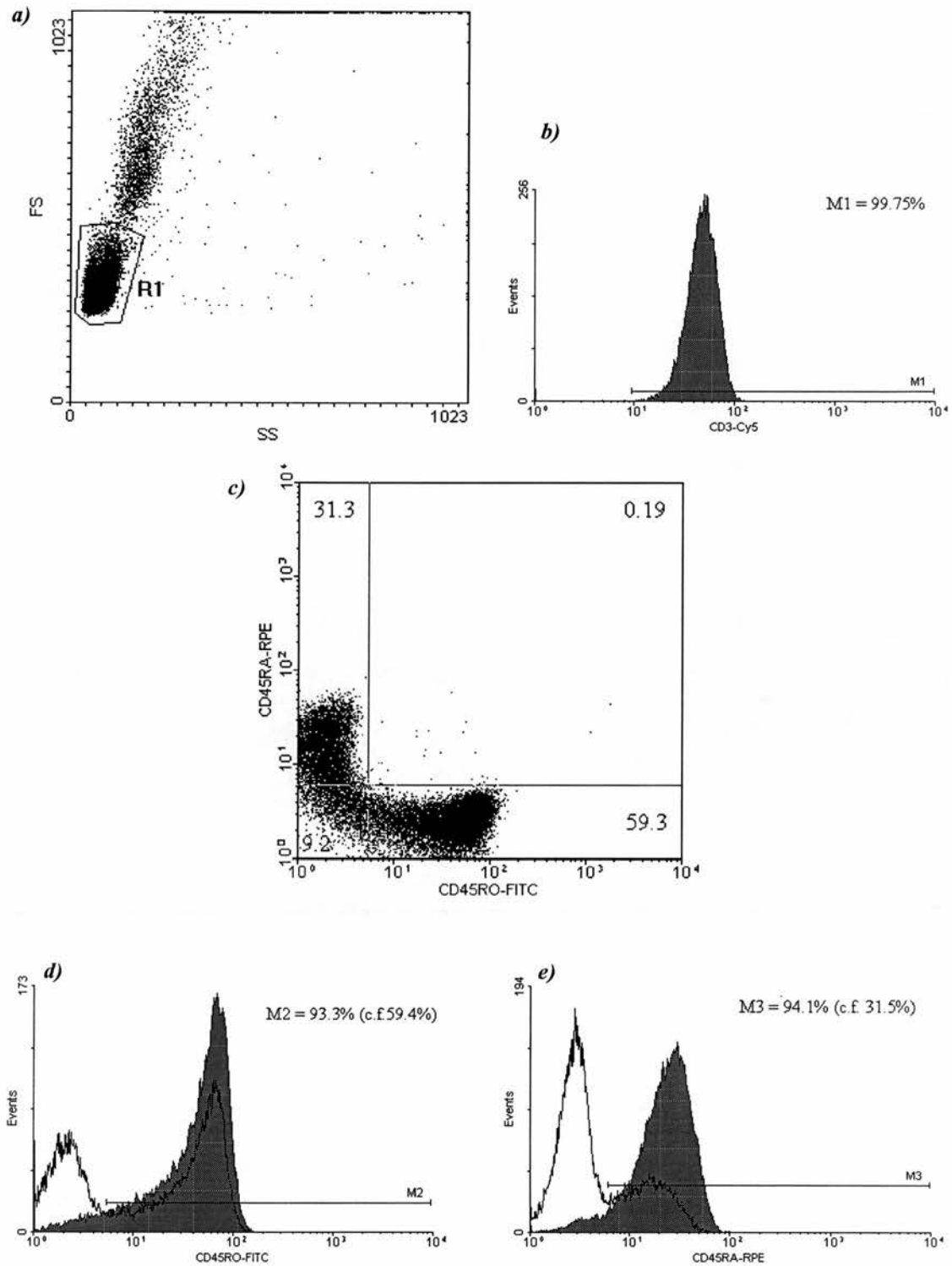

FIGURE 20. *Flow cytometric analysis of CD45RA$^+$ and CD45RO$^+$ cells: a) FS/SS of CD4$^+$ cells before negative selection — region R1 was used as a lymphocyte gate, b) M1 was used to gate CD3$^+$ cells within R1, c) distribution of CD45 isotypes by double staining of unseparated CD4$^+$ cells, d) CD45RO expression after depletion of CD45RA$^+$ cells (shaded), e) CD45RA expression after depletion of CD45RO$^+$ cells (shaded) — in each case, the outlined histogram shows the distribution of the same marker before negative selection.*

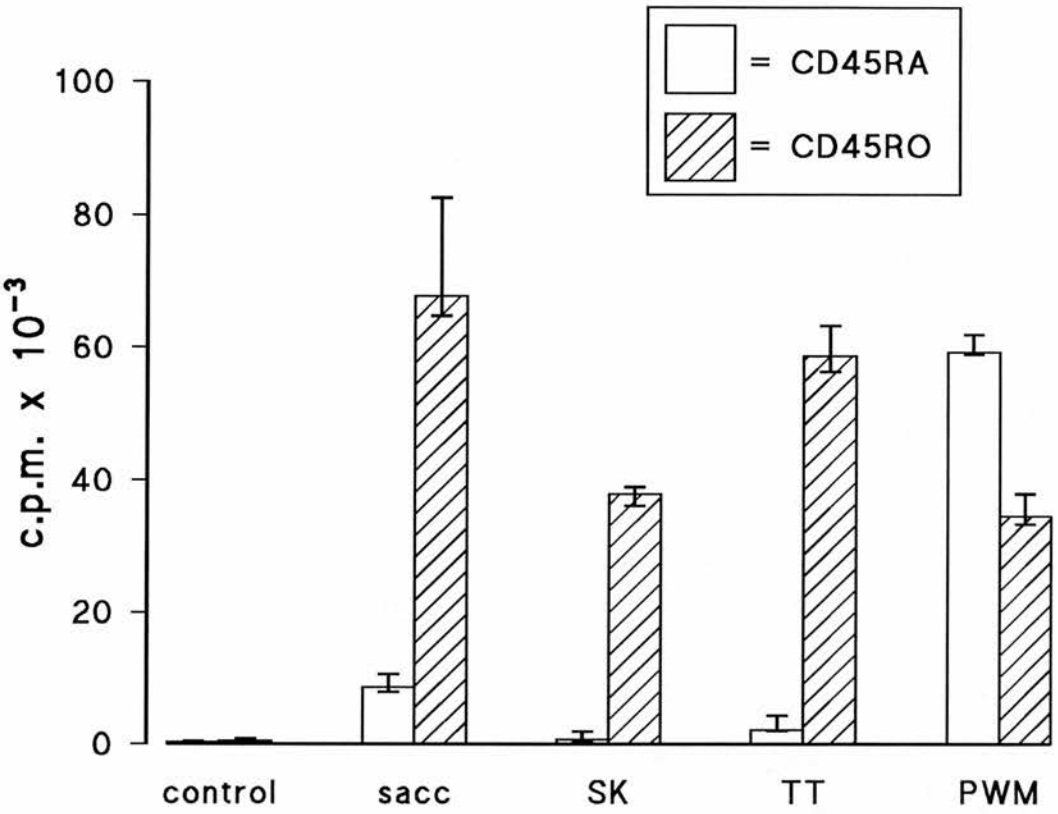

FIGURE 21. *Response of CD45RA$^+$ and CD45RO$^+$ cells to sacc, SK, TT and PWM. Bars represent median and range of five replicate counts.*

STIMULUS	CD45RA		CD45RO	
control	294	—	509	—
sacc	8,590	(29.2)	67,730	(133)
SK/SD	714	(2.4)	37,883	(74.4)
TT	2,126	(7.2)	58,722	(115)
PWM	59,294	(202)	34,548	(67.9)

TABLE 12. *Data from Fig. 21. Numbers are median counts per minute of five replicates, with stimulation indices in brackets.*

Precursor cell frequency

SACC-SPECIFIC PRECURSORS

Measurement of the frequency of sacc-specific cells in the circulating population was attempted in one normal donor. A preliminary experiment was performed, using semilog increments in T-cell (as E^+ cells) input, in order to guide further experiments (Fig. 22a). Three further attempts were then made to measure the precursor frequency, with various cell inputs and numbers of replicates. However, in all except the first experiment, χ^2 analysis suggested that the null hypothesis of single-hit kinetics should be rejected (Fig. 22b, c & d). Notwithstanding this, the calculated frequencies were compatible between experiments.

SK-SPECIFIC PRECURSORS

A single experiment to measure SK-specific precursor cell frequency did conform to single-hit kinetics and gave a result very similar to that for sacc (Fig. 23).

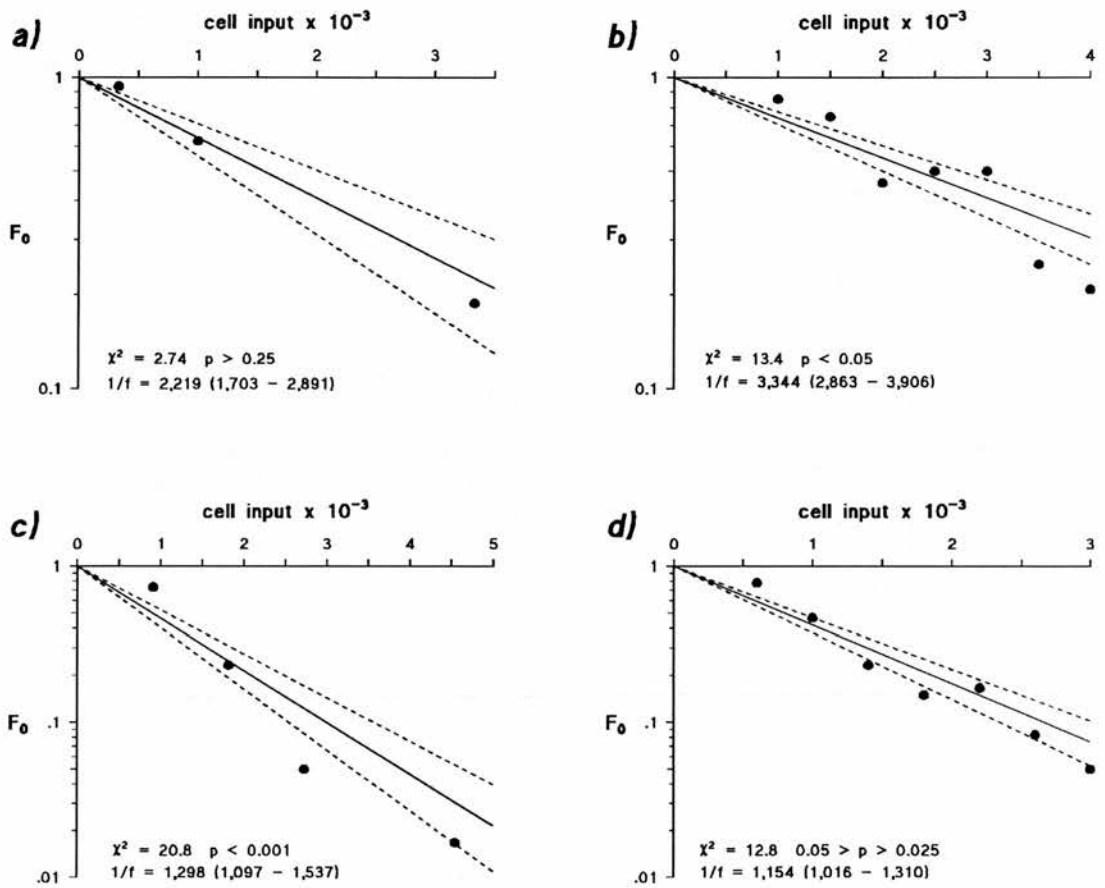

FIGURE 22. *Sacc-specific precursor cell frequency. Lines represent the calculated precursor frequency and its 95% confidence interval. For each plot, the relevant χ^2 and associated P values are shown with the reciprocal precursor frequency and its 95% confidence interval. Forty-eight replicates were used at each cell input for 'a' and 'b', and sixty for 'c' and 'd'. (In each case, the degrees of freedom are one fewer than the number of data points.)*

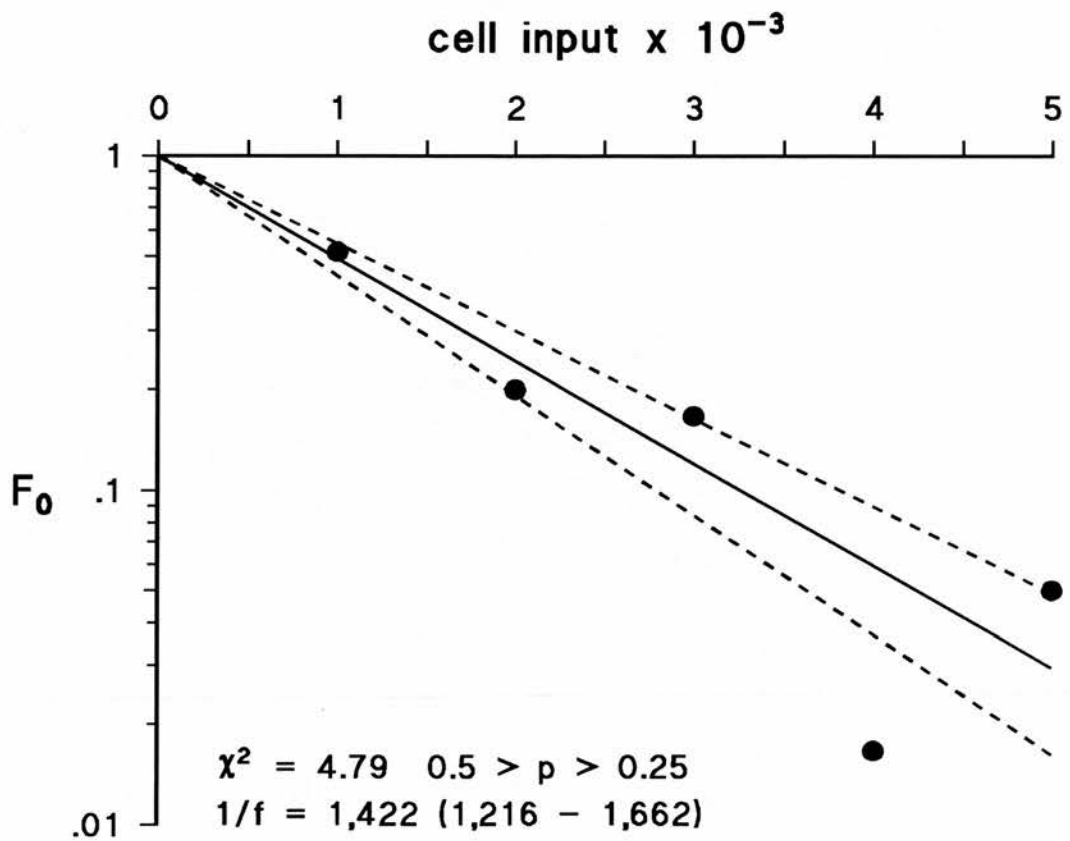

FIGURE 23. *SK-specific precursor cell frequency. Treatment as for Fig. 22. Sixty replicates were used at each cell input.*

Cytotoxicity of stimulated PBMC

The % cytotoxicity of PBMC, obtained from six normal donors and cultured in the presence of different stimuli, against the mel-1 and molt-4 cell lines is shown in Fig. 24. The effector to target cell ratio of the data shown was 10:1 (similar results were obtained for ratios of 20:1 and 5:1 but these data were less complete). All stimulated effector cells demonstrated significantly greater cytotoxicity against each of the target cell lines than did the unstimulated control cells ($P < 0.05$ in all cases; Wilcoxon signed rank test). Antigen- and sacc-stimulated cells elicited greater killing against the NK-sensitive line (molt-4) compared with the NK-resistant line (mel-1).

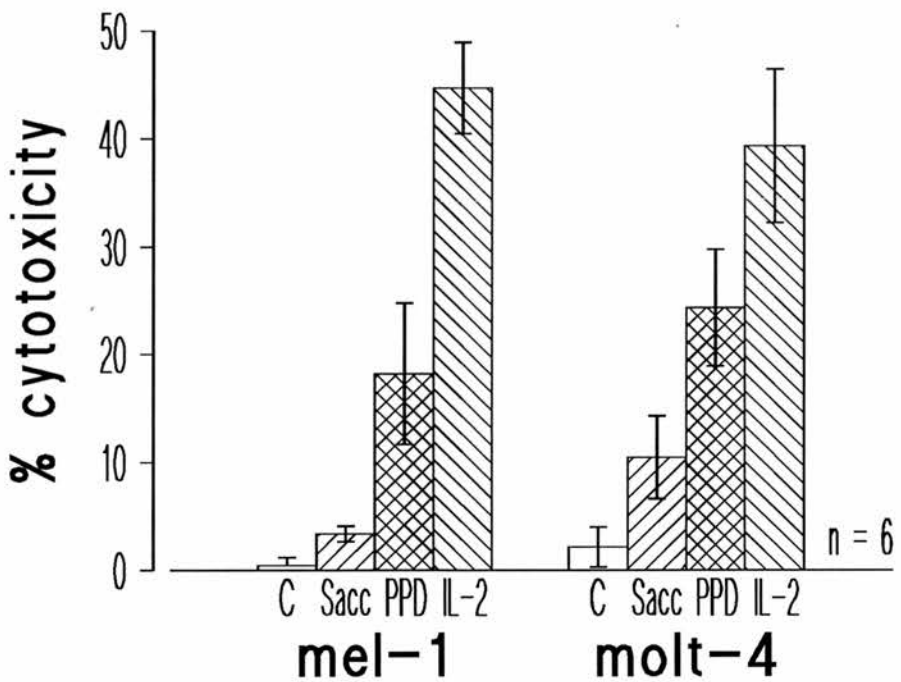

FIGURE 24. *Cytotoxicity assay. Sacc and PPD were used at the concentrations which gave optimal stimulation in the proliferation assay; IL-2 was at 50 u/ml. Bars show mean ± SEM.*

T-cell lines and clones

PHA CLONES

Seven clones grew successfully following stimulation with PHA and IL-2; five were $CD4^+$ and two $CD8^+$, by flow cytometry. All retained the ability to respond to the mitogens, PHA and PWM, but, with the exception of one $CD8^+$ clone (JD.R4), which appeared to show a small response to PPD, none proliferated in response to sacc or PPD (Fig. 25).

SACC-SPECIFIC LINES AND CLONES

After repeated rounds of stimulation with sacc, IL-2 and APC in bulk culture, it was possible to produce a T-cell line which appeared to demonstrate specificity for sacc.

A single $CD4^+$ clone which also demonstrated sacc-specificity was isolated by limiting dilution at a frequency of 3 cells/well (Fig. 26).

However, it was not possible to reproduce these results after further stimulation.

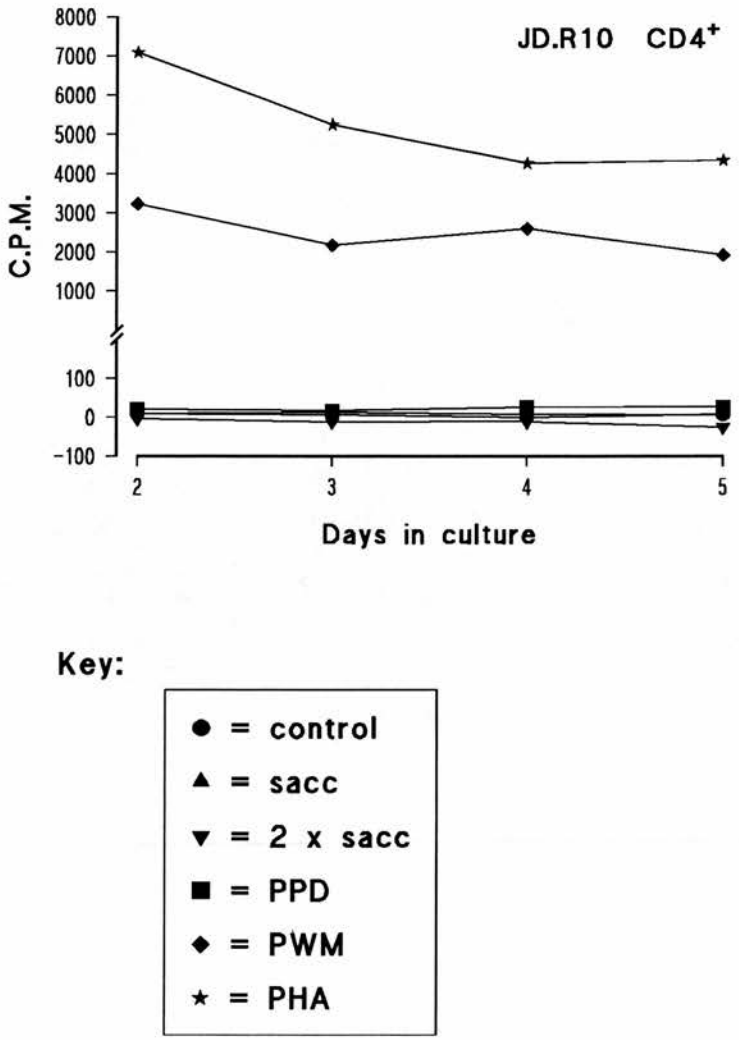

FIGURE 25. *Proliferation responses of clones initiated with PHA/IL-2. Clones were cultured at 5,000 cells/well with 10^5 irradiated PBMC as APC. Points are medians of four replicate counts. Negative values are a consequence of subtraction of [^{3}H]-Tdr uptake of irradiated PBMC in the absence of responder cells. 'Sacc' and '2 × sacc' represent protein concentrations of 8.6 and 17.2 μg/ml, respectively (see Fig. 9).*

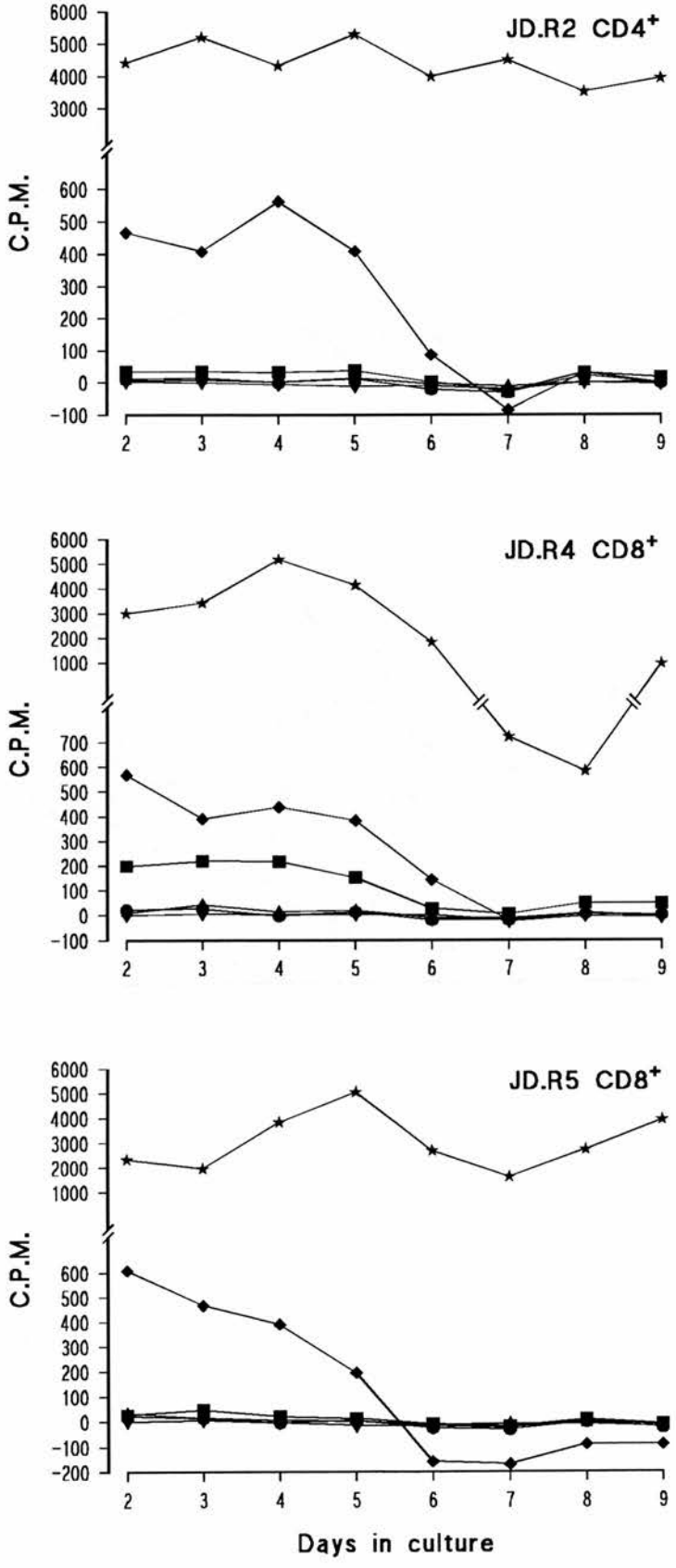

FIGURE 25. *cont.*

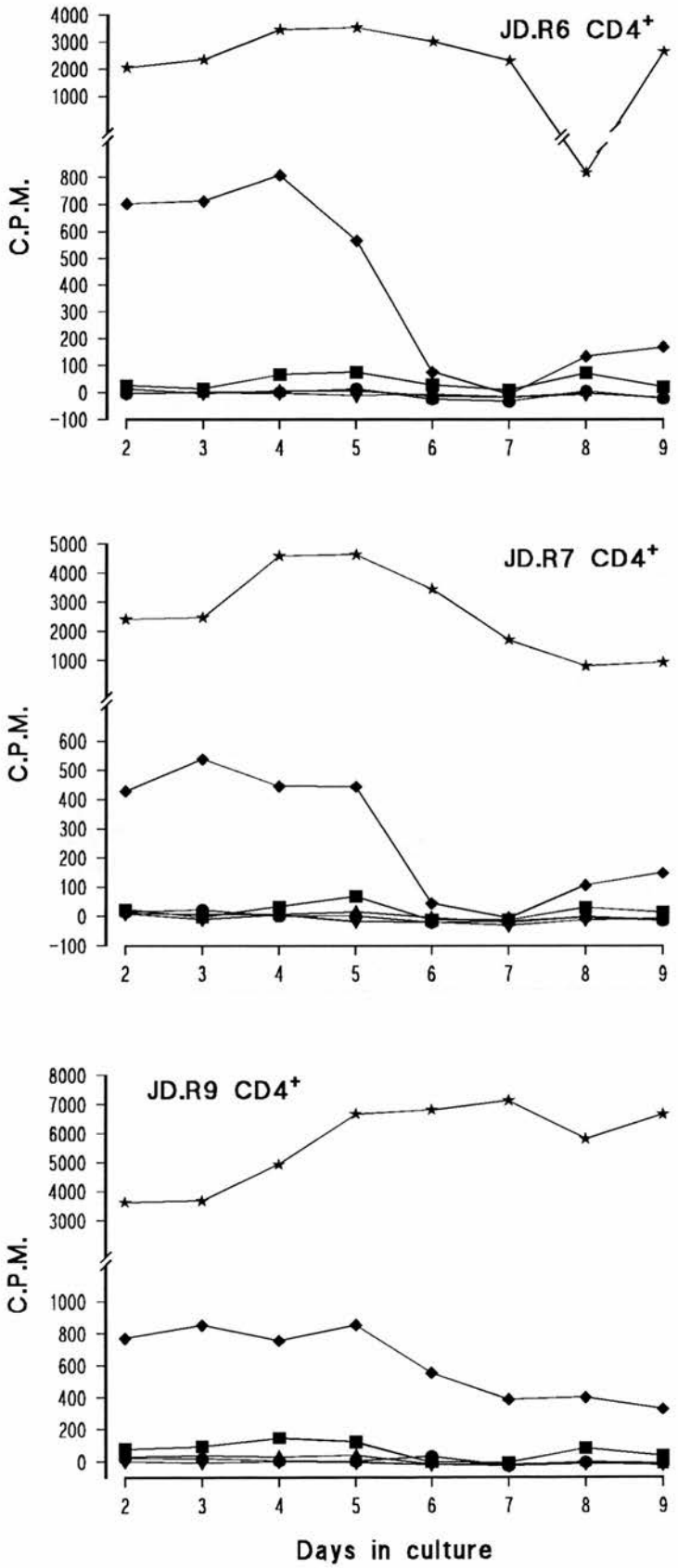

FIGURE 25. *cont.*

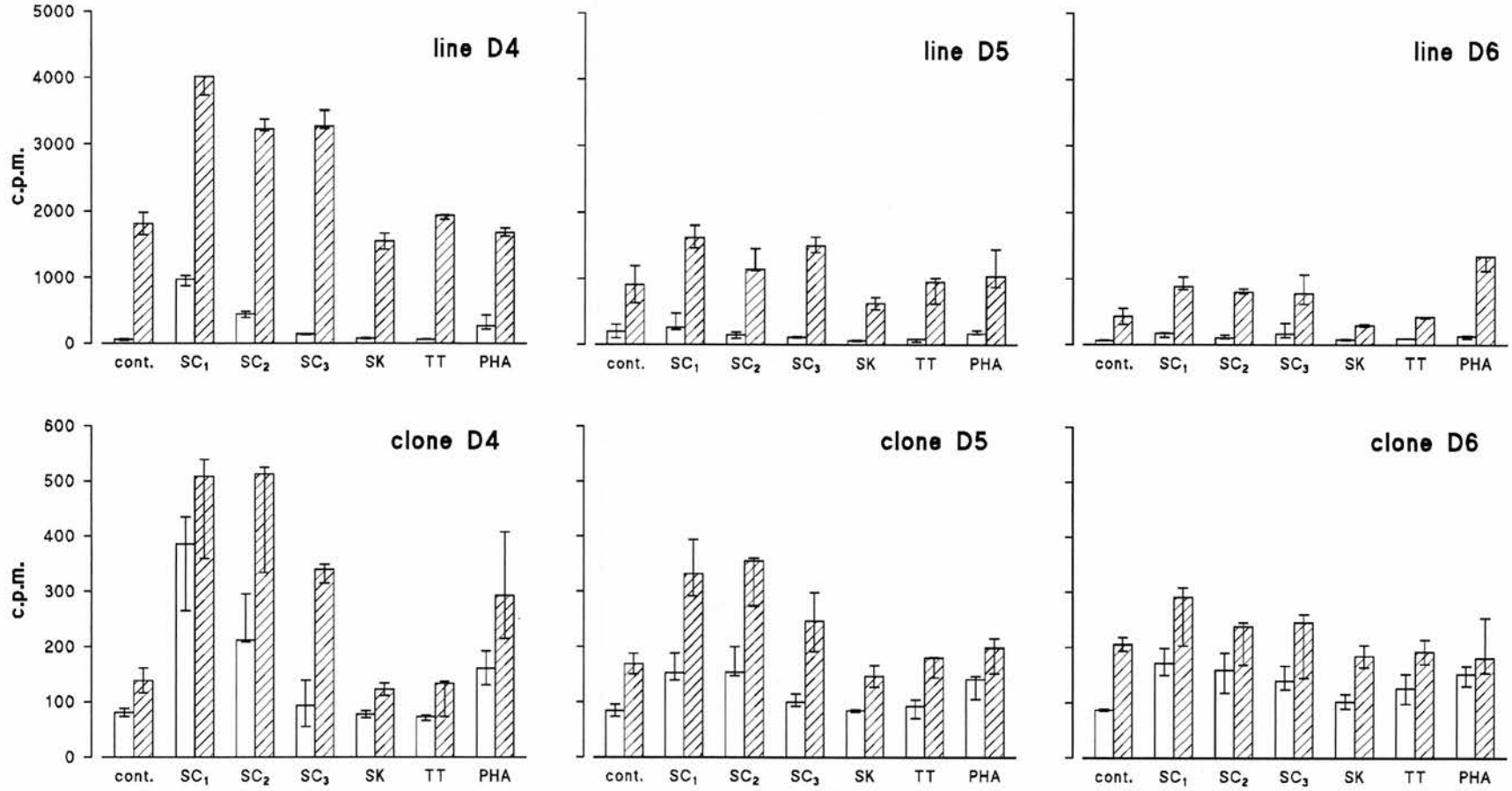

Figure 26. *Proliferative response of sacc-specific T-cell line and clone. Cultures included 5,000 responders/well with 2 × 10^4 E^- cells as APC. Responses are shown at three different times and in the presence (shaded bars), or absence (open bars) of IL-2 at a suboptimal concentration of 4 u/ml. SC_2 represents the concentration of sacc used to initiate the cultures; SC_1 and SC_3 are, respectively, three times and one third of that concentration. Bars show median and range of three replicate counts.*

Cellular response to sacc in Crohn's disease

As before, IgG anti-sacc was greater in the Crohn's disease group than in the normal controls (Crohn's: 2.4, 1.10–4.40; normal: 0.71, 0.51–1.04; $P = 0.009$) (Fig. 27). These values were lower than in the equivalent groups in the antibody study described in Part I; however, all of these samples were processed in a single assay and so would have been equally affected by any systematic error. The mean serum AAG of the Crohn's group was 1.20 g/l (1.04–1.37), which was very similar to that of the larger sample reported previously.

Several significant differences were found between groups when comparing the sacc-specific proliferative responses at different doses (Fig. 28). However, in view of the number of comparisons made, these significance values should be interpreted with caution, and they do not convey a strong impression of a real difference between the Crohn's disease and normal groups.

There were no significant differences between the two groups (either in absolute c.p.m. or S.I.) in the proliferative response to any of the concentrations of SK, TT or PPD tested (Fig. 29).

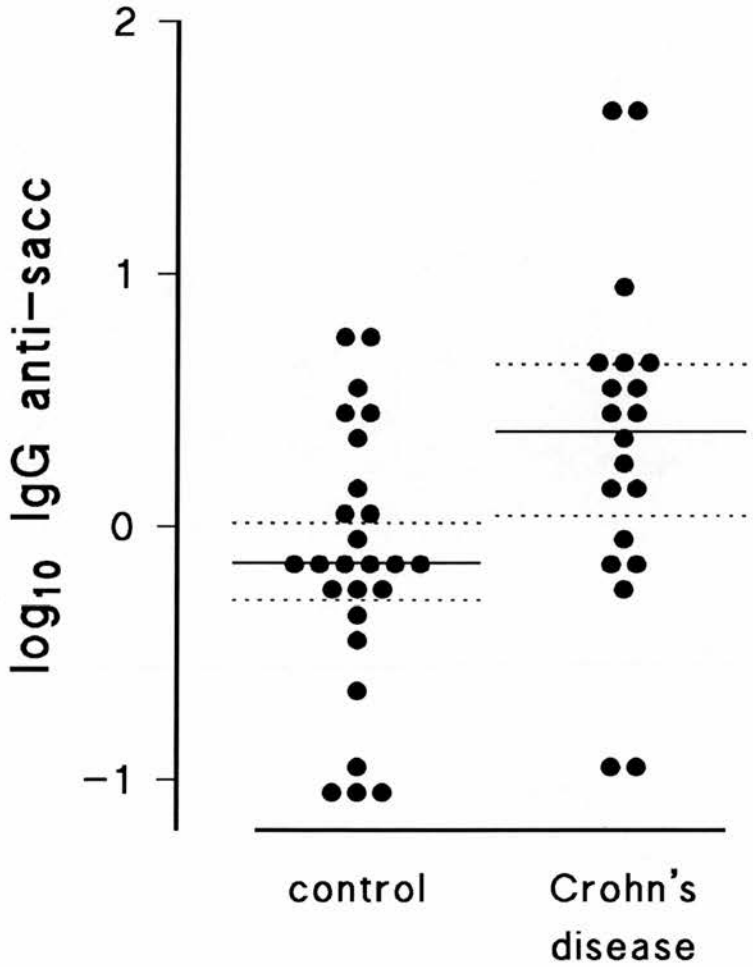

FIGURE 27. *IgG anti-sacc in Crohn's disease and normal controls. Data have been log transformed and grouped at intervals of 0.1. Lines show medians (solid) and 95% C.I. (dashed), calculated on the untransformed data.*

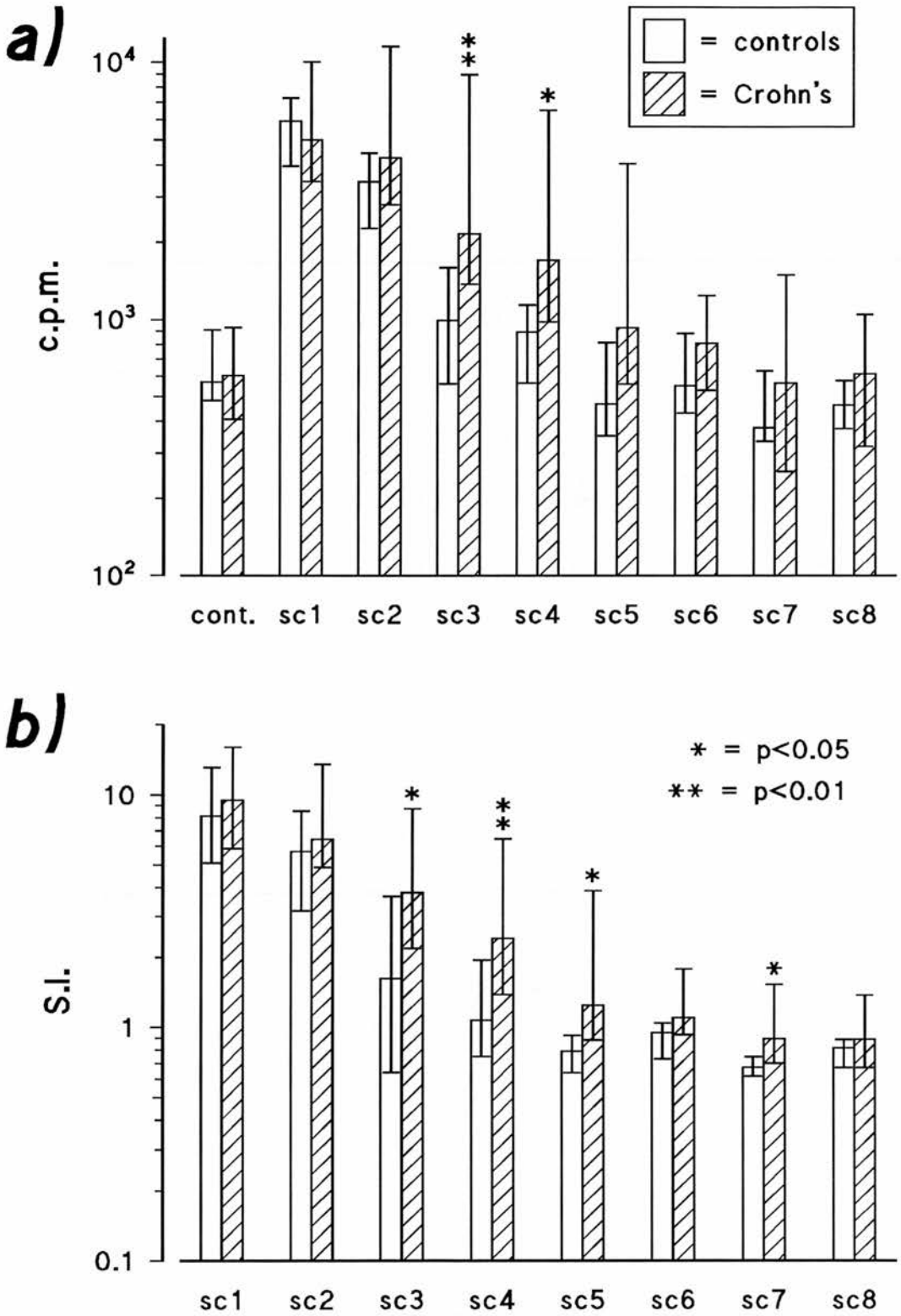

FIGURE 28. *Proliferative response to sacc in Crohn's disease and normal controls. a) c.p.m., b) S.I. Bars show 95% confidence intervals about medians of six replicates. SC_1 represents the optimal stimulating concentration of sacc; SC_2–SC_8 are successive semilog dilutions.*

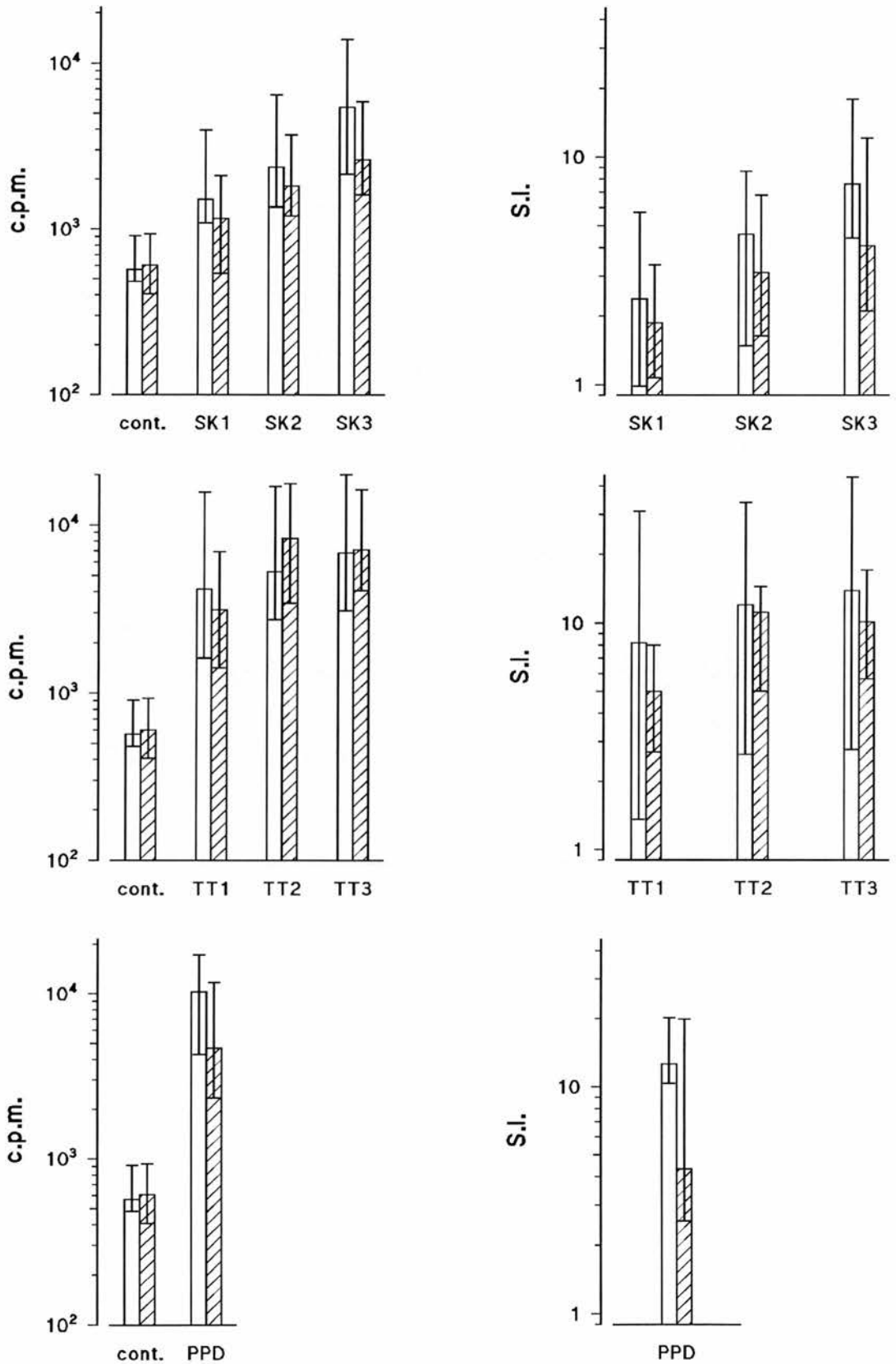

FIGURE 29. *Proliferative response to SK, TT and PPD in Crohn's disease (shaded) and normal controls (open). Bars show 95% confidence intervals about medians of six replicates. SK_1, TT_1 and PPD are optimal stimulating doses, with semilog dilutions of SK and TT as in Fig. 28.*

III

Observations on the nature of sacc antigen

MATERIALS AND METHODS

Fractionation of sacc

ULTRAFILTRATION AND GEL FILTRATION

A purified high molecular weight fraction was prepared[a] by subjecting the aqueous extract to an approximately six-fold volume reduction by ultrafiltration using a TCF 2 micro-thin-channel ultrafiltration system *(Amicon)* with membranes having a 50 kD molecular weight cut-off; the ultrafiltrate was then lyophilised and the re-dissolved product was applied to a Sepharose CL6B column *(Pharmacia)* equilibrated in sterile water. The fraction eluting in the void volume was collected and lyophilised. This fraction showed a single band of apparent MW = 200 kD after SDS-PAGE and staining with Coomassie brilliant blue and PAS.

PROLIFERATIVE RESPONSE TO HIGH MOLECULAR WEIGHT FRACTION

PBMC, obtained from four donors who had previously demonstrated significant proliferative responses to sacc, were tested for their ability to proliferate

a: this preparation was the work of Mrs. S. Allen.

in response to doubling dilutions of the high molecular weight fraction.

Effect of pronase on sacc

PRONASE DIGESTION

A stock solution of sacc was prepared by dissolving the lyophilised preparation at 100 mg/ml in PBS, centrifuging to remove undissolved material, and sterile filtering to 0.2 μm.

A stock solution of pronase (Sigma) at 100 mg/ml was prepared by adding 10 ml PBS to a bottle containing 1 g of the enzyme. Further dilutions, with pronase concentrations of 0.2 and 2 mg/ml, were prepared in PBS.

The following mixtures were prepared in sterile tubes, using *equal volumes* of the solutions indicated:

A:	sacc	+	PBS
B:	sacc	+	0.2 mg/ml pronase
C:	sacc	+	2.0 mg/ml pronase
D:	PBS	+	0.2 mg/ml pronase
E:	PBS	+	2.0 mg/ml pronase

The tubes were incubated at 37 °C for 24 hours, heated in a boiling water bath for 15 minutes, cooled to room temperature, centrifuged to remove any precipitate, and the supernatants recovered. These were then mixed as follows, again using equal volumes throughout:

α:	A	+	PBS
β:	B	+	PBS
γ:	C	+	PBS

δ:	D	+	PBS
ε:	E	+	PBS
ζ:	A	+	D
η:	A	+	E

Thus, preparations were derived which contained: sacc which had been subjected to 'mock' treatment with incubation and heat only (α); sacc treated with pronase at 0.1 and 1.0 mg/ml before heat-inactivation (β and γ, respectively); incubated, heat-inactivated pronase only (δ and ε); and mock-treated sacc to which incubated, heat-inactivated pronase had been added (ζ and η). Constant dilution factors were used throughout to ensure the equivalence of the actively treated preparations and their respective controls.

MEASUREMENT OF CARBOHYDRATE CONCENTRATION

Carbohydrate concentration, as total monosaccharide, was measured colorimetrically by the phenol/sulphuric acid method of DuBois.[244]

Solutions of α-D-glucose and α-D-mannose *(Sigma)*, at 10 mg/ml in distilled water, were prepared for use as standards, and stored at −30 °C until required. The assay was performed by making doubling dilutions of the standard solutions and test preparations, adding 200 μl of each to 200 μl water-saturated phenol, and then rapidly adding 1 ml concentrated sulphuric acid. The light absorbance of the resulting solutions was measured at a wavelength of 492 nm (using an ELISA plate-reader to measure duplicate 200 μl aliquots in 96-well plates), and estimates of the carbohydrate concentrations of different dilutions of the test solutions were derived with reference to a titration curve compiled from the standards, the form of the

calculation being exactly as described for the ELISA techniques previously discussed.

MEASUREMENT OF PROTEIN CONCENTRATION

Protein concentration was measured colorimetrically by the Bradford reaction method.[245]

Solutions of bovine serum albumin *(Sigma)*, at 10 mg/ml in distilled water, were prepared for use as standards, and stored at −30 °C until required. Coomassie brilliant blue dye concentrate *(BioRad)*, was diluted 1:4 v/v with distilled water. The assay was performed by making doubling dilutions of the standard and test solutions, adding 40 µl of each to 2 ml dye, and allowing 30 minutes for full colour development. The light absorbance of the resulting solutions was measured relative to a 'reagent blank' (40 µl distilled water + 2 ml dye), at a wavelength of 595 nm (using a spectrophotometer and disposable polystyrene cuvettes), and estimates of the protein concentrations of different dilutions of the test solutions were derived with reference to a titration curve compiled from the standards, as above.

PROLIFERATION RESPONSE TO PRONASE DIGEST

PBMC derived from a normal donor (known to be responsive to sacc in this assay) were used. Serial dilutions of preparations α-η were made in complete culture medium at dilutions of 1/8, 1/24, 1/80, 1/240, 1/800 and 1/2,400 and 50 µl of each added to five replicate wells containing 10^5 cells in 150 µl.

Cells were pulsed with [^{3}H]-Tdr for six hours on day 6, before harvesting and β-counting.

ELISA INHIBITION

The effect of treatment with pronase on the ability of sacc to inhibit specific antibody-binding in the quantitative ELISA was assessed.

Serial 3-fold dilutions of preparations α-η were made in PBS, and 40 μl of each were then added to 220 μl aliquots of the pooled standard IgG anti-sacc serum, the latter having been pre-diluted in PBS-Tween such that the final serum dilution, after addition of the inhibitor, was 1/500; 40 μl of PBS were added to diluted serum as controls. The test and control samples were incubated for 1 hour at room temperature and then assayed, in duplicate, for IgG anti-sacc antibody by ELISA.

For each pair of duplicate observations a *fractional inhibition* was calculated according to:

$$\frac{\text{test mean} - \text{control mean}}{\text{control mean}} \times 100\%$$

Inhibition curves, showing fractional inhibition as a function of the dilution factor, were then constructed for each preparation.

Comparison of sacc with mannan preparations

PREPARATION OF 'CRUDE' MANNAN

A mannan preparation was made according to Sutherland and Wilkinson.[246] Dried yeast was suspended in distilled water at 200 g in 1 l, washed three times and resuspended in 500 ml 0.05 M citrate buffer, pH 7. After heating in a boiling water bath for 1–2 hours and allowing to cool, the suspension was centrifuged at $10^4 \times g$ for 15 min. and the supernatant retained. Following the addition of 25 ml glacial acetic acid, centrifugation was repeated to remove the resulting precipitate and the

supernatant was neutralised with 6 M NaOH. Mannan was then precipitated and washed by adding 1 l ethanol at −40 °C and collecting in a Buchner funnel.

PURIFIED MANNAN

Purified mannan, prepared from *S. cerevisiae* by the cetavlon method, was obtained from a commercial source *(Sigma)*.

Solutions of sacc, crude mannan and purified mannan were prepared in distilled water at 100 mg/ml, filtered, and stored at −30 °C. Carbohydrate and protein analysis, lymphoproliferative response and ELISA inhibition were assessed as above.

RESULTS

High molecular weight fraction of sacc

Proliferative response

Cells from all of the donors demonstrated a proliferative responses to this preparation after 6, 7 or 8 days in culture (Fig 30).

Pronase digestion of sacc

Effect on protein and carbohydrate content

The titration curves obtained from the carbohydrate and protein standards are shown in Fig. 31 & 32, respectively. Also plotted are the absorbance values obtained from the pronase-treated and control preparations. Results calculated from these data are given in Table 13 & 14, respectively.

Active treatment with pronase did not differ from control treatments in its effect on carbohydrate content, which was ~8.4 mg/ml (equivalent to 33.6 mg/ml in the original stock solution of sacc) in all the preparations containing sacc, except that treated with 1.0 mg/ml pronase, in which the calculated mean concentration may have been unduly influenced by a single 'outlier' (Fig. 31 & Table 13).

In the protein assay, sacc behaved differently from the bovine serum albumin standard, giving a titration curve which was markedly non-parallel to the standard curve. However, active treatment with pronase, at both 0.1 and 1.0 mg/ml, reduced the protein content to below the minimum which the assay could detect (~250 μg/ml), whereas the control-treated preparations gave titrations which were identical to each other, resulting in estimated protein concentrations of 1.16–2.01 mg/ml (equivalent to 4.65–8.03 mg/ml in the stock sacc solution) (Fig. 32 & Table 14).

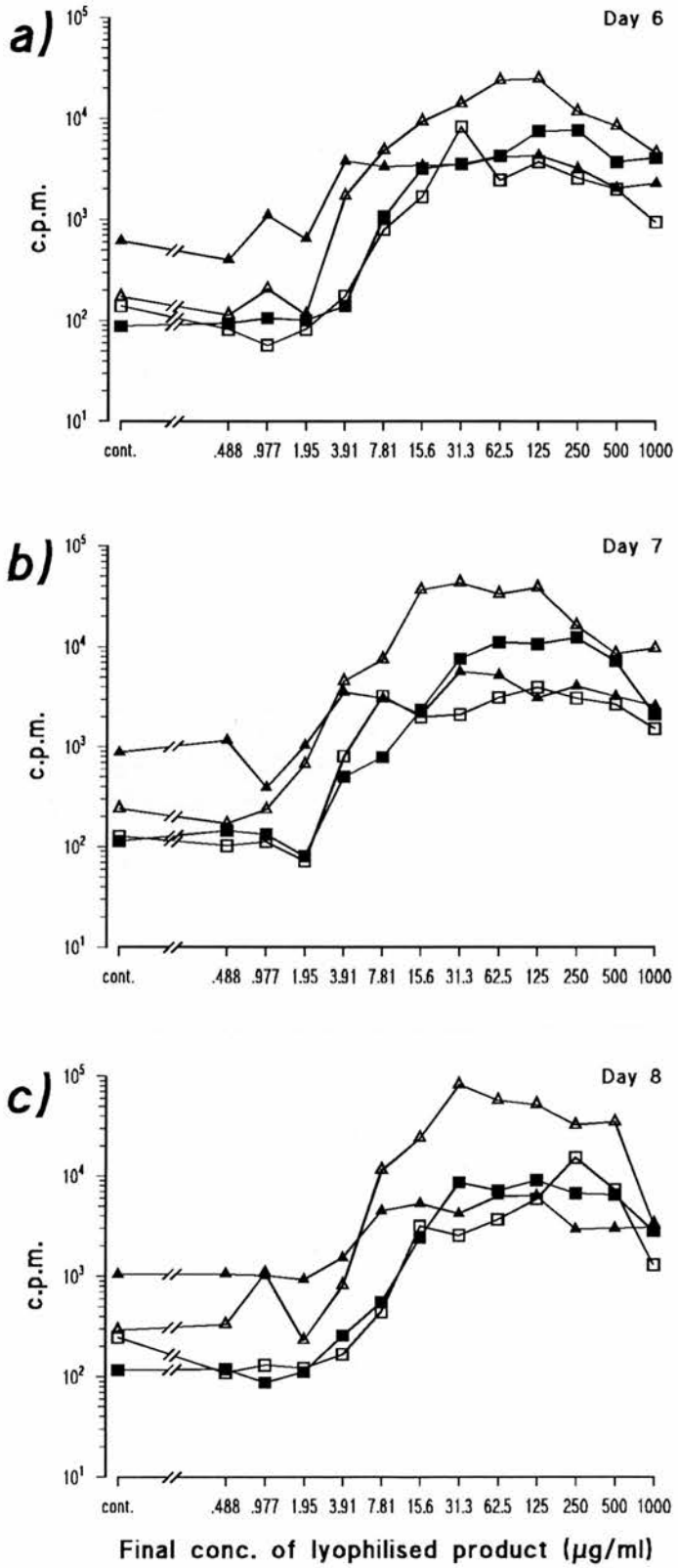

FIGURE 30. *Proliferative response to high molecular weight fraction of sacc. Data points are medians of five replicates. Symbols represent different donors.*

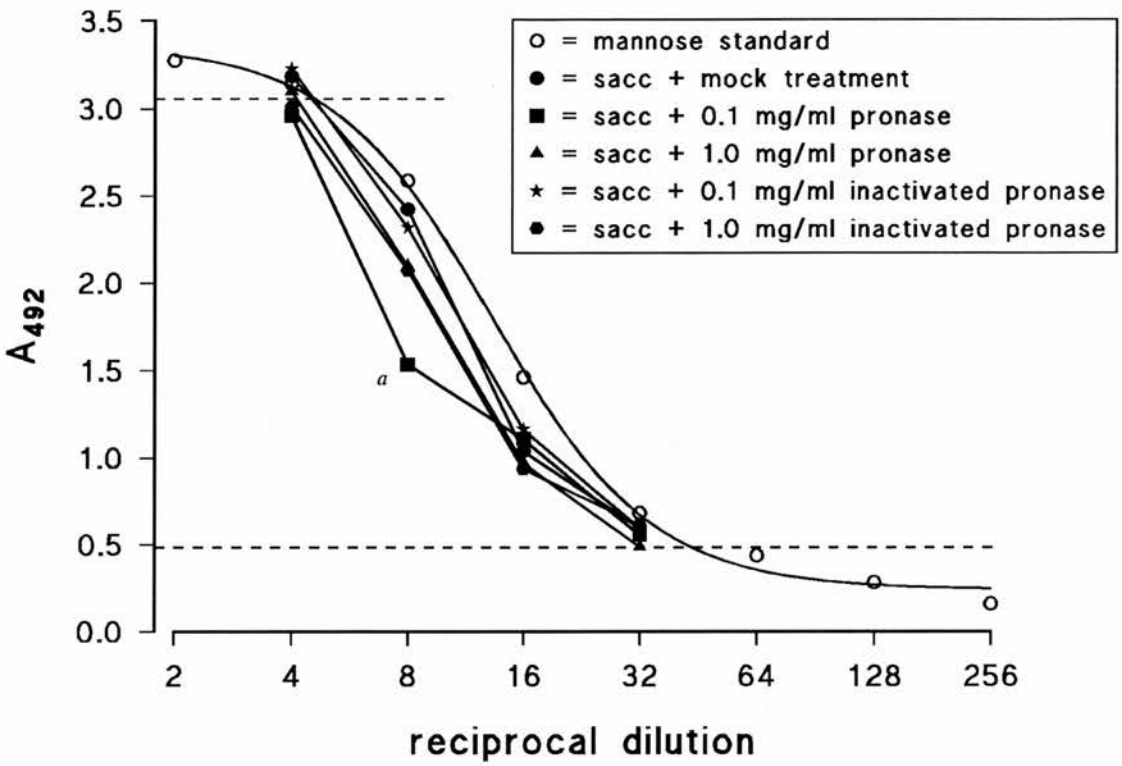

FIGURE 31. *Effect of pronase and control treatments on carbohydrate content of sacc. The dashed lines delineate the portion of the standard titration curve used to calculate results. Carbohydrate was not detected in preparations containing inactivated pronase alone (not shown).*

a: see text and Table 13.

PREPARATION	CARBOHYDRATE CONCENTRATION (mg/ml)	DILUTION AT WHICH MEASURED	MEAN CARBOHYDRATE CONCENTRATION (mg/ml)
sacc + mock treatment	9.02	1/8	
	7.20	1/16	8.4
	8.98	1/32	
sacc + 0.1 mg/ml pronase	9.41	1/4	
	5.09[a]	1/8	7.6
	7.60	1/16	
	8.40	1/32	
sacc + 1.0 mg/ml pronase	11.86	1/4	
	7.23	1/8	8.3
	6.79	1/16	
	7.39	1/32	
sacc + 0.1 mg/ml inactivated pronase	8.37	1/8	
	7.93	1/16	8.4
	8.96	1/32	
sacc + 1.0 mg/ml inactivated pronase	10.17	1/4	
	7.12	1/8	8.3
	6.61	1/16	
	9.25	1/32	

TABLE 13. *Effect of pronase and control treatments on carbohydrate content of sacc. The concentrations have been derived from the data shown in Fig. 31.*

a: this concentration is derived from a point which appears to be an outlier in Fig. 31. Its exclusion results in a mean concentration of 8.5 mg/ml.

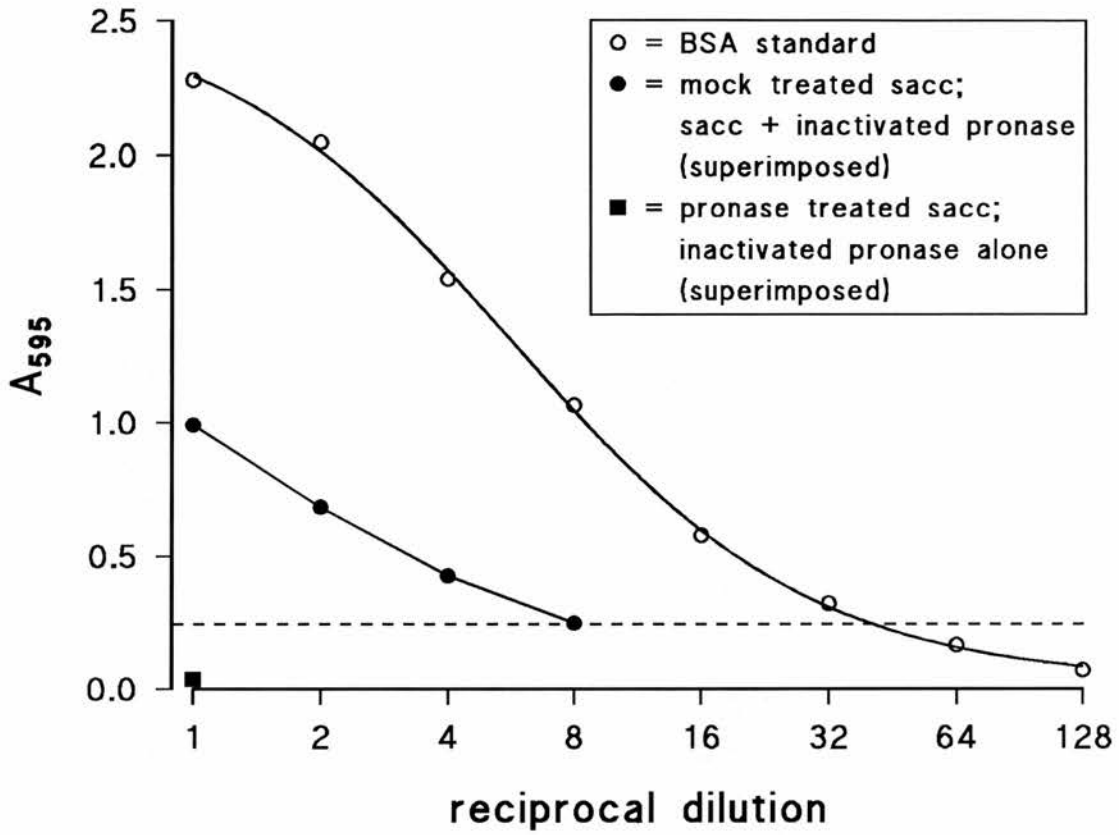

FIGURE 32. *Effect of pronase and control treatments on protein content of sacc. The dashed line demarcates the lowest absorbance value used to calculate results.*

PREPARATION	PROTEIN CONCENTRATION mg/ml	DILUTION AT WHICH MEASURED	MEAN PROTEIN CONCENTRATION mg/ml
sacc + mock treatment	1.16	1	
	1.46	1/2	1.59 (geometric mean = 1.26)
	1.74	1/4	
	2.01	1/8	

TABLE 14. *Protein content of sacc subjected to control treatments. The concentrations have been derived from the data shown in Fig. 32. The data shown are for mock treatment, but those for the other control treatments are indistinguishable.*

Effect on proliferation response

The effect of pronase digestion on the sacc-dependent lymphoproliferative response is shown in Fig. 33.

Treatment with 1.0 mg/ml pronase completely abolished thymidine uptake by peripheral blood lymphocytes, although some proliferation was still evident with 0.1 mg/ml pronase (Fig. 33a). Treatment with inactivated pronase demonstrated no clear effect (Fig. 33b) and there was no proliferative response to the heat-inactivated enzyme alone (Fig. 33c).

Effect on ELISA inhibition

The results of the ELISA inhibition assay are shown in Fig. 34.

There was an approximate two-fold concentration difference between the inhibition curves obtained with actively treated sacc and that obtained with mock treated sacc. However, a similar result was obtained with sacc to which 1.0 mg/ml inactivated pronase had been added. Therefore, the observed effect is likely to be due either to experimental variation or an effect of the pronase protein independent of its enzymatic activity. In any case, the effect was much less than that seen in the ability of pronase treatment to abrogate the T-cell response to sacc. No inhibition was observed with inactivated pronase alone.

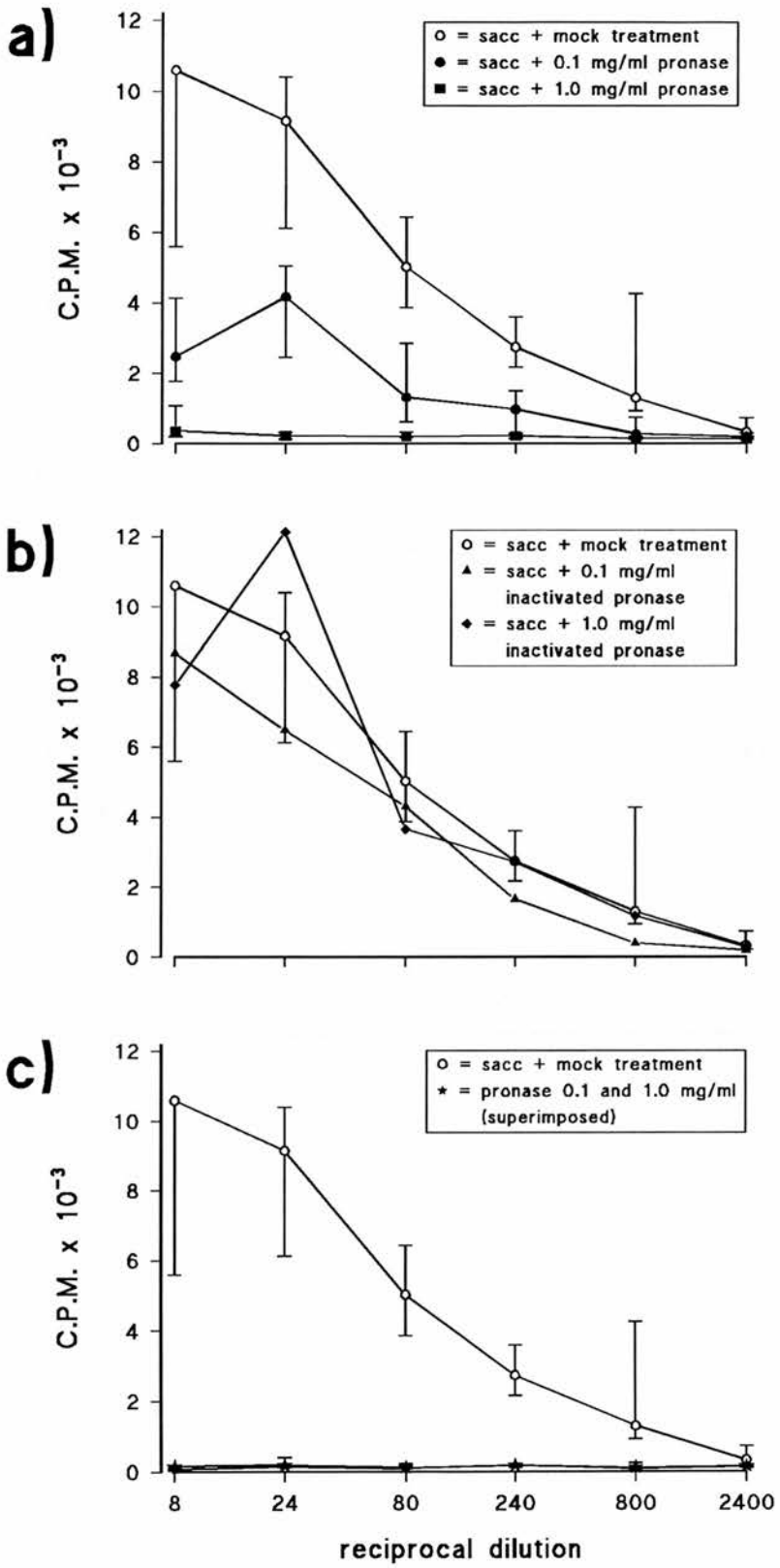

FIGURE 33. *Effect of pronase treatment on the lymphocyte proliferation response to sacc. Data points are the medians of five replicates and error bars show ranges (for clarity, these are omitted from two of the data sets in 'b').*

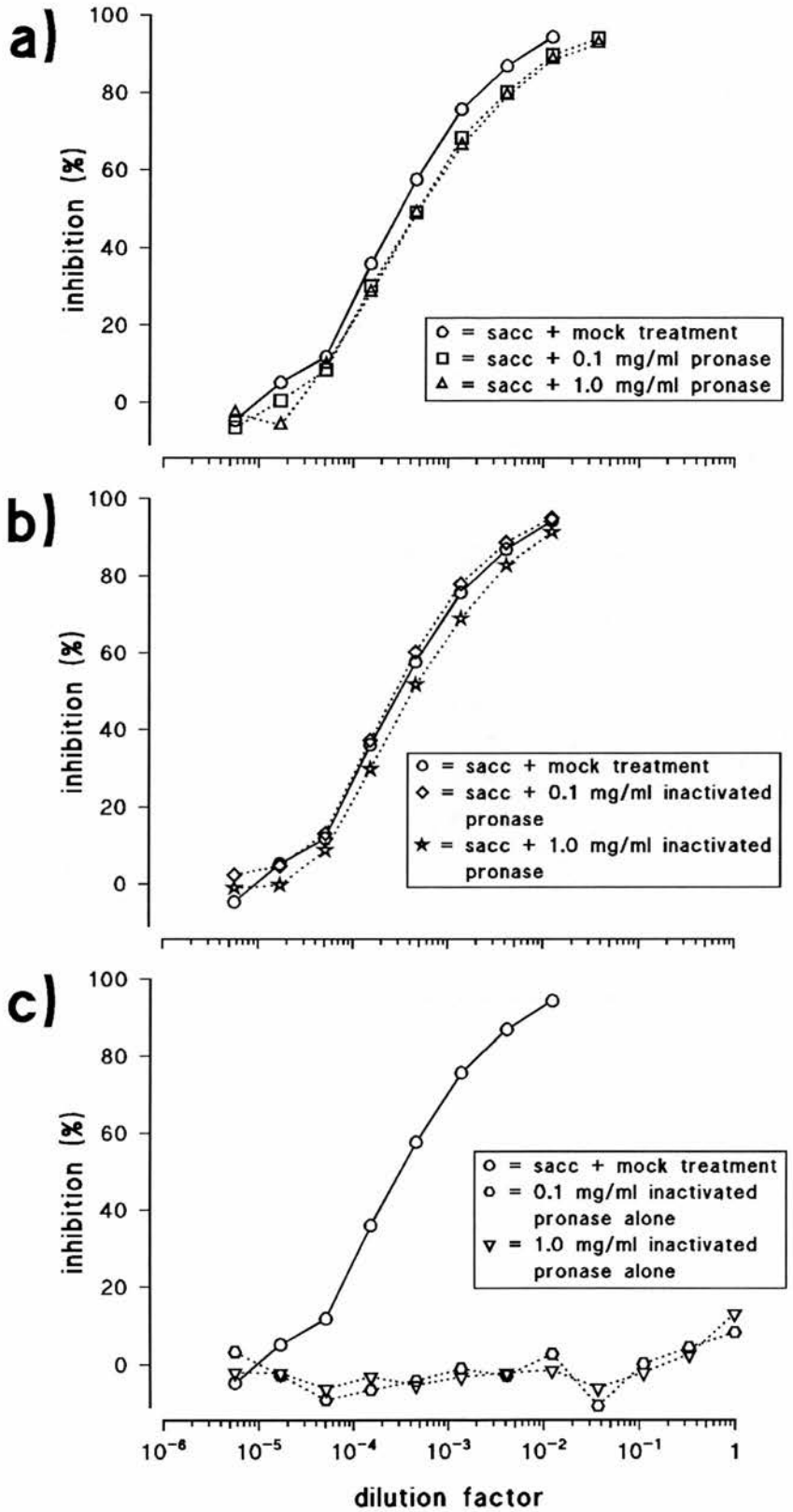

FIGURE 34. *ELISA inhibition by pronase-treated sacc. The ability of mock-treated sacc in the fluid phase to compete for antibody binding with sacc on the solid phase is compared with that of: a) sacc treated with active pronase, b) sacc treated with inactivated pronase, c) inactivated pronase alone.*

Comparison of sacc with mannan preparations

PROTEIN AND CARBOHYDRATE ANALYSIS

The titration curves obtained in the protein and carbohydrate assays of these preparations are shown in Fig. 35 & 36, and the data calculated from these in Table 15. In the case of the purified mannan, virtually all of the dissolved material was accounted for as carbohydrate. However, in the case of sacc and the crude mannan, a considerable proportion of the original material was unaccounted for — an observation explained by the fact that when the solutions of these preparations were made, it was noted that visible amounts of undissolved material were invariably present; therefore, the actual final concentration of total solute would have been less than the 100 mg/ml intended. Nevertheless, the ratio of protein to carbohydrate in the crude mannan was reduced relative to that of sacc.

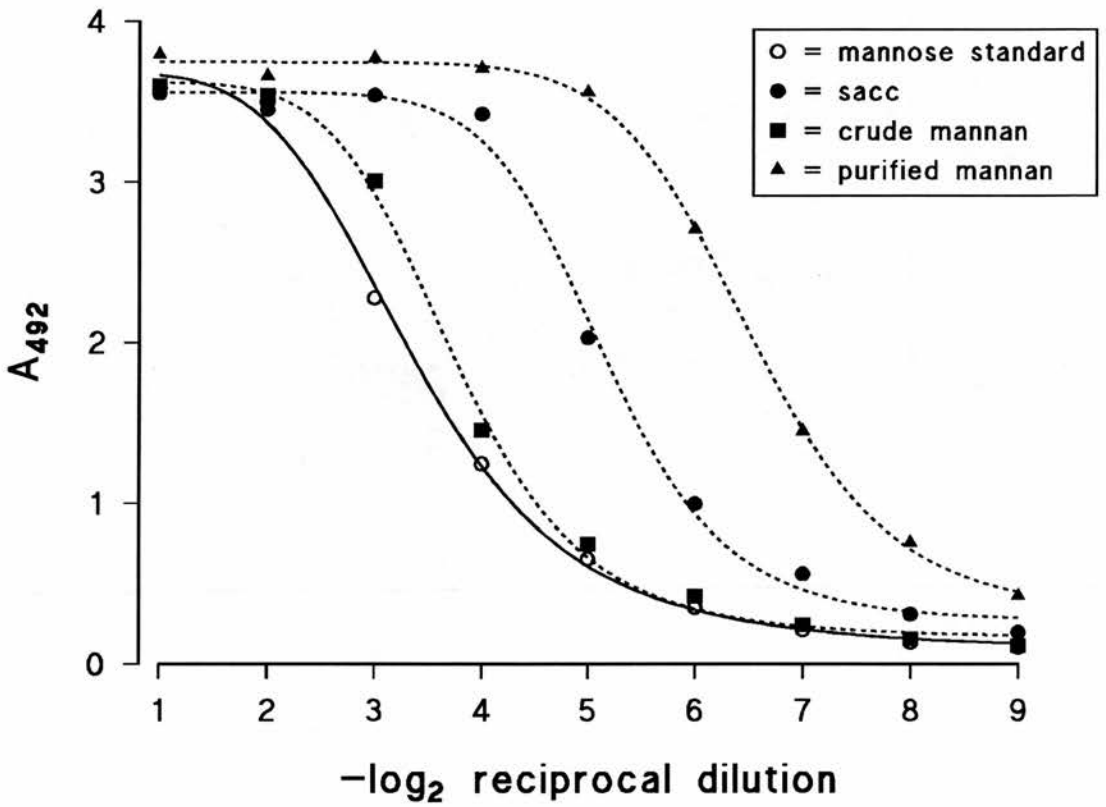

FIGURE 35. *Carbohydrate assay of sacc and mannans. Test solutions were at 100 mg/ml and the standard at 10 mg/ml.*

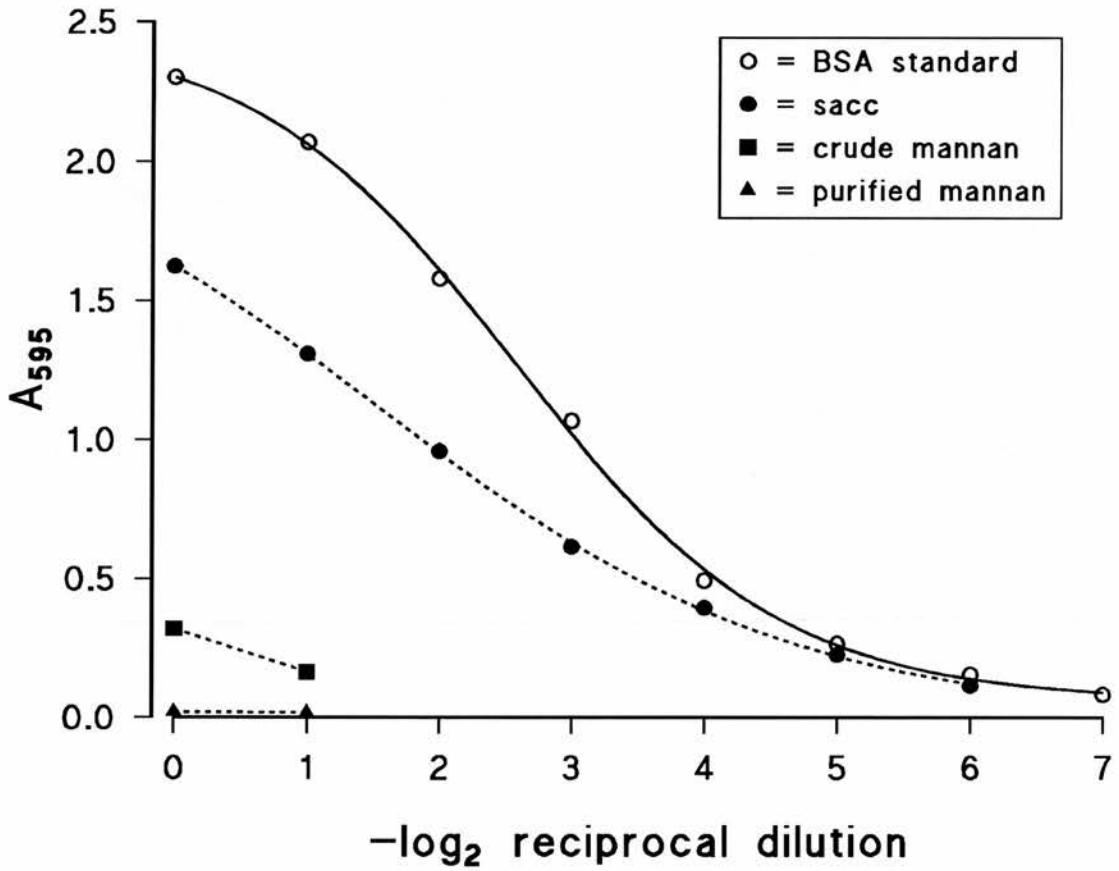

FIGURE 36. *Protein assay of sacc and mannans. Test solutions were at 100 mg/ml and the standard at 10 mg/ml.*

Preparation	Carbohydrate concentration (mg/ml)	Dilution at which measured	Mean carbohydrate concentration (mg/ml)	Protein concentration (mg/ml)	Dilution at which measured	Mean protein concentration (mg/ml)
Sacc	34	1/32		2.54	1	
	32.4	1/64	33.8	3.49	1/2	4.78 (geometric mean = 4.46)
	35	1/128		4.63	1/4	
				5.76	1/8	
				7.48	1/16	
Crude mannan	14.5	1/8		0.38	1	—
	11.9	1/16	12.7			
	12.0	1/32				
	12.4	1/64				
Commercial mannan	97.8	1/64		< 0.36	—	—
	95	1/128	96.6			
	97	1/256				

Table 15. *Protein and carbohydrate content of sacc and mannans. Data are derived from Fig. 35 & 36.*

LYMPHOCYTE PROLIFERATION

The PBMC response to the crude mannan was inferior to that of sacc at an equivalent dilution (Fig. 37). However, if the dilution factor of the crude mannan is corrected for protein content relative to sacc, and the response re-plotted, it approaches that obtained with sacc (Fig. 37, inset) *{Note: correction for protein was based on the relative displacement of the curves obtained with sacc and crude mannan, without reference to the BSA standard curve}*. There was no detectable uptake of [^{3}H]-Tdr in response to the purified mannan.

ELISA INHIBITION

The crude mannan elicited a similar degree of inhibition to sacc in this assay. The relationship was even closer when the dilution factor of the crude mannan was corrected for carbohydrate content relative to sacc, and the inhibition curves re-plotted (Fig. 38 and inset).

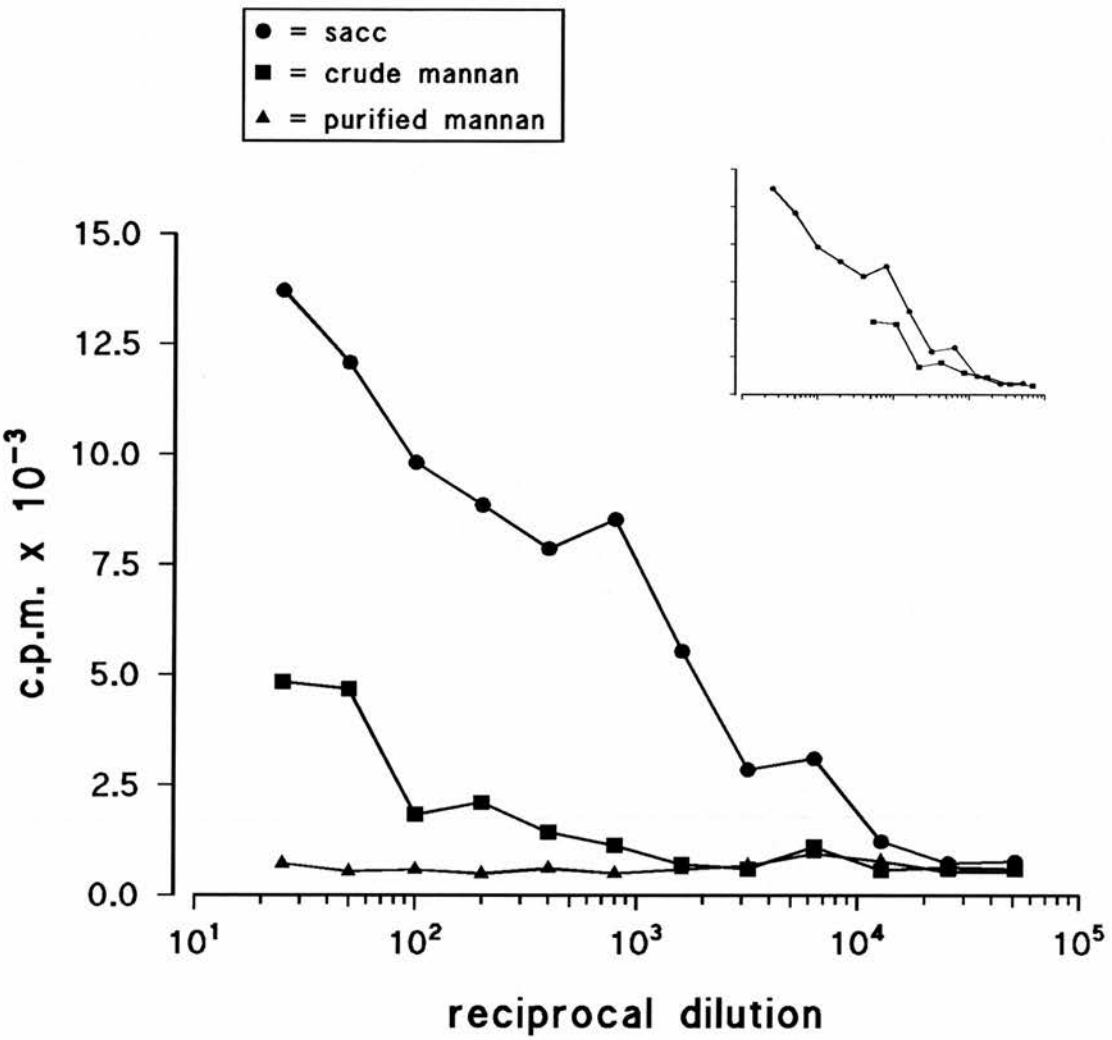

FIGURE 37. *Proliferative response to sacc and mannans. Data points are medians of five replicates.* **Inset:** *response to sacc and crude mannan re-plotted after correcting dilution factor of the latter for protein content.*

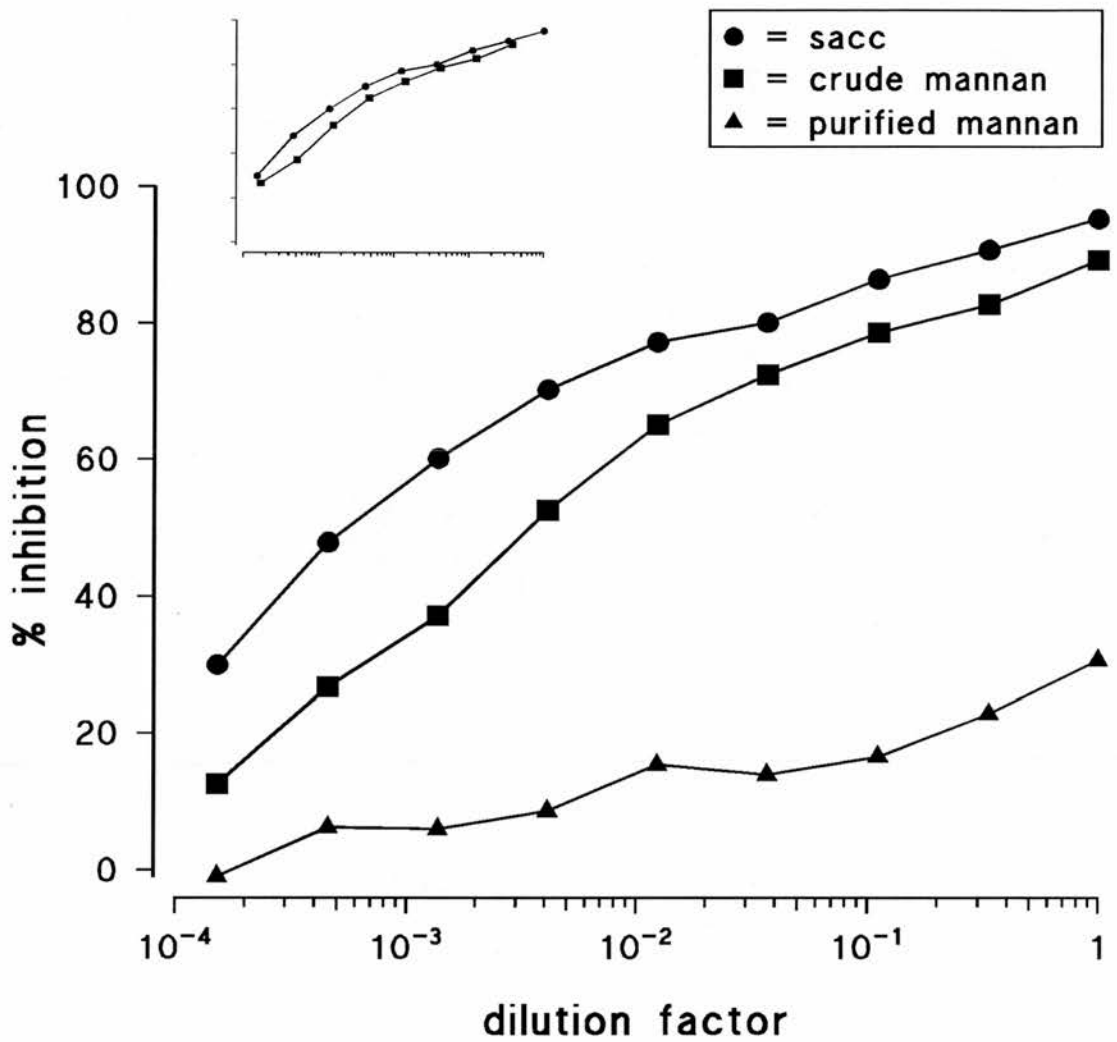

FIGURE 38. *ELISA inhibition with sacc and mannans. Data points are means of duplicates.* **Inset:** *inhibition due to sacc and crude mannan re-plotted after correcting dilution factor of the latter for carbohydrate content.*

DISCUSSION

Antibodies to sacc in gastrointestinal disease

Because previous studies of antibodies to *S. cerevisiae* in inflammatory bowel disease all used sub-optimal methods of internal standardisation and/or quantitation, I have attempted to develop fully quantitative, isotype-specific ELISAs, using an aqueous extract of yeast as the antigen. By screening a number of sera, it was possible to find examples with high antibody-binding, which were suitable for pooling together to provide standard calibrants. A 'control' assay, for measuring IgG anti-casein, was developed in parallel. The assays thus derived all had very low intra-assay variation, and the inter-assay coefficient of variation was less than 10%, except in the case of the IgG anti-sacc ELISA, where a storage artefact may have resulted in an erroneously high estimate of 15–19%.

I wished to determine whether the results of previous studies could be reproduced with this assay. In addition, it was desirable to obtain further information on the influence of site and activity of Crohn's disease on antibody levels, since this has either been omitted from earlier reports, or data have been conflicting.

Although the different groups in this study were not matched for age and sex distribution, and there was a significant bias towards males amongst healthy subjects, the antibody results in this group were not dependent on either of these variables. The median value of IgG anti-sacc in patients with Crohn's disease was over three times that in healthy subjects. A smaller increase was seen in patients with chronic liver disease of various aetiologies. The latter group was included in this study because there was pre-existing evidence of enhanced antibody responses to other dietary

antigens in liver disease (see p. 13); it was therefore felt that these patients would be a valuable control group, when assessing whether enhanced systemic immune responsiveness to yeast was specific for Crohn's disease.

The results for IgA show that only partial success was achieved in respect of the aim of obtaining fully quantitative results with the antibody assays. This was because specific IgA was unmeasurably low in a large proportion of samples from all of the groups tested — only 20% of sera from normal subjects could be quantified for IgA anti-sacc; even among sera from Crohn's disease patients (who had the highest proportion of quantifiable sera), only 55% could be quantified. It may have been possible to 'push' the assay by using lower dilutions of the test sera, but this was avoided for technical reasons.[152] Therefore, IgA results were analysed after classification as 'positive' or 'negative'. The threshold level chosen as the basis for this classification was the 95th. centile of the values obtained from normal sera, the rationale for the choice being that this would result in an acceptably low number of 'false positives' if the assay were to be used as a diagnostic test, as well as allowing a reasonably robust test of significance. IgA anti-sacc was positive in one third of Crohn's disease patients,[a] this frequency being over six times that in normal subjects. In patients with liver disease, there was also an increased frequency of positive results for IgA, but the *P* value achieved in this case was not sufficiently low for the difference to remain significant after taking into account the number of comparisons.

Among patients with Crohn's disease, there was a strong trend towards higher levels of IgG anti-sacc in those with small bowel disease (with or without large bowel disease) compared to those without small bowel disease, but this did not quite achieve

a: applying a similar analysis to IgG anti-sacc gave a result of ~45%

significance. IgA anti-sacc correlated with duration of disease, when the actual numerical value of the former was taken into account, and IgG anti-sacc was higher in patients in whom the serum AAG was above the normal range.

These results confirm that the humoral response to *S. cerevisiae* is enhanced in Crohn's disease, but they also show that the finding is not confined to this group, being also a feature of chronic liver disease. The absence of an increased response in subjects with coeliac disease was not unexpected, since all were on a gluten-free diet and evidence suggests that antibodies to non-gluten dietary antigens may decline under these conditions (see p. 12, 13). The results also suggest, but do not establish, a relationship between antibodies to sacc and disease of the small bowel, active disease and disease of longer duration. It is important to note, however, that the Crohn's disease group consisted virtually exclusively of outpatients with relatively well-controlled disease (median AAG of 1.1 g/l and median *Bristol Activity Index* score 1.5). Therefore, the power of the study to detect any effect due to this variable will have been low. Furthermore, in the majority of cases, the symptoms and/or diagnosis had been established for several years (median duration 7 years), and so no information is available pertaining to antibody levels at or near the time of onset of disease. A prospective study would be a suitable way of investigating whether antibody measurements made as early as possible after presentation might have any predictive value in diagnosis.

There is clearly a question to be asked concerning the mechanism of the increased antibody response in Crohn's disease. It is not possible to say whether the production of anti-sacc antibodies reflects an immune response which is generated following sensitisation of cells of the gastrointestinal lymphoid system *per se*, or

whether it represents a systemic response to antigen which has 'bypassed' the normal handling mechanisms of the gut and gained access to the systemic circulation. However, activation of lamina propria T-cells is evident in active Crohn's lesions,[247] and it is conceivable that at least some of these are recognising peptide products of luminal antigens. Furthermore, Peyer's patches, which are specialised sites for sampling and processing antigen, are predominantly localised in the small bowel,[248] which could explain an association of the antibody response with disease at this site, were one to be confirmed. Conversely, evidence has been available for many years that intact protein can be absorbed from normal mucosal surfaces in sufficient quantities as to be biologically significant, as demonstrated experimentally by the phenomenon of passive cutaneous anaphylaxis,[249] and clinically by systemic anaphylaxis to foods in sensitised subjects.

Neither the Crohn's disease nor liver disease group differed from healthy subjects with respect to IgG anti-casein. This protein was chosen as a control antigen because Barnes *et al.*[19] found that the majority of sera with anti-milk antibodies reacted with it, although other studies have more frequently found antibodies to other milk proteins (see p. 6 *ff*). In any case, the choice was effective since a wide range of binding activities could readily be detected and quantified in normal and pathological samples. It is therefore interesting to speculate that the enhanced antibody response to *S. cerevisiae* in Crohn's disease may be dependent on peculiarities of its antigenic structure.

Finally, it should be borne in mind that no inference can be taken from these data as to the biological significance of anti-sacc antibodies, nor concerning any possible aetiological role of bakers' yeast in Crohn's disease. Although it is well-

established that the condition responds to therapeutic strategies based on replacement of the normal diet by various liquid feeds,[250] and, in one study, yeast was identified subjectively by patients as having contributed to symptomatic deterioration,[251] there is only one published investigation of the effect of yeast exclusion on disease activity. Barclay *et al.*[252] used a Crohn's disease activity index (CDAI) to monitor the progress of 19 patients who were in clinical remission at the start of the study. Following a baseline observation period, all patients ate a low-yeast diet to which placebo and yeast capsules were added (these were given sequentially and the order was randomised). The mean CDAI was higher during the yeast inclusion period than the yeast exclusion period. This was also true for the maximum CDAI, which was also lower during the yeast exclusion period than the baseline period. However, the differences, though significant, were very small.

Thus, although this study adds to the evidence that Crohn's disease patients are predisposed to increased antibody responses to some dietary antigens, there is currently no reason to presume that this is other than an epiphenomenon of the disease process.

Cellular responses to sacc

In this study, a proliferative response of PBMC to sacc was universally detectable in all healthy subjects. The magnitude of the response, however, was very variable between individuals. In this respect, it was similar to the response to the recall antigen PPD. In addition, maximal *in vitro* responses to the optimal stimulating concentrations of sacc and PPD occurred after about one week in culture; this was in contrast with the earlier response to the polyclonal mitogen, PWM. Maximal tritiated

thymidine uptake due to PHA also occurs early, at about day 3 (not shown).

In order to characterise the immunological response to sacc, it was important to establish whether it was acting as a true antigen or as a non-specific lymphocyte activator. My first approach to this problem was to test whether sacc could induce proliferation in cord blood lymphocytes. Cord blood contains immature lymphocytes which have been said to be relatively unresponsive to various stimuli.[253] With respect to *Candida* mannan, a negative response of CBMC in one study has been claimed as evidence for antigen-specificity,[145] whereas a positive response in another was considered to be evidence for mitogenicity.[254] Similarly, mitogenicity was inferred from CBMC responsiveness to a cell wall/membrane preparation from *Cryptococcus neoformans*.[255] I found the most striking feature of CBMC to be a very high rate of thymidine uptake in the absence of any stimulus. This observation, which has been made by some authors[153,253] but not others[256] is likely to have had a profound effect on the SIs, since the denominator in the calculation is necessarily large. Some authors have quoted only SIs as measures of CBMC responses,[255] but this study clearly shows that uncritical acceptance of this derived variable could be problematic. In fact, visual inspection of the cultures suggested that only PHA and PWM resulted in true proliferation, suggesting that the high background counts in these cells may relate to 'redundant' DNA synthesis uncoupled to proliferation *per se*. In any case, the contention that CBMC do not contain cells capable of antigen-specific responses is not unassailable, since cells responding to hsp 65 have been reported to be present at a precursor frequency similar to that in adult PBMC, and PPD-specific precursors at a frequency of as high as 1/6,000.[222] Furthermore, MHC class II-dependent CBMC responses to PPD and *Paracoccidioides brasiliensis* have been reported,[256] and CBMC

responses to dietary antigens has been claimed to predict the development of future allergy.[257,258]

Stronger evidence of antigen-specificity came from experiments using purified cell populations. In the first of these, T-cells were isolated by sheep cell rosetting and further depleted of adherent cells; non-rosetting cells were used as APC or accessory cells. T-cells depleted of APC were profoundly unresponsive to sacc and the recall antigens SK and TT. The response to PHA was less sensitive to depletion of accessory cells (the fact that some stimulation was still detected implies that the 'T-cell' preparation still contained some accessory cells, since a pure T-cell population cannot respond even to PHA[259]). Furthermore, responsiveness to sacc was restored incrementally by the addition of increasing numbers of APC. All responses were abrogated by irradiation of the T-cell fraction, but not the APCs, confirming that the former were the responding cells.

When cells from three healthy donors were incubated with sacc, SK or TT, there was a rise in the proportion of $CD3^+$ cells expressing the CD4 co-receptor molecule and a reciprocal fall in $CD8^+$ cells, compared with unstimulated cells or cells incubated with PHA. These changes were more marked among cells with high forward- and side-scatter, which are likely to include blasts. A further experiment, using highly purified $CD4^+$ and $CD8^+$ cells from one of these donors showed that only $CD4^+$ cells responded to sacc, SK and TT, whereas both populations responded to PHA.

CD45 (leucocyte common antigen) is a transmembrane tyrosine phosphatase involved in signal transduction which is expressed in multiple isoforms through alternative splicing of three exons encoding *N*-terminal sequences.[260] The observation

that only T-cells expressing the low molecular weight CD45RO isoform have the capacity to respond to recall antigens and provide help for antibody production led to the proposal that CD45RO was a marker for the 'memory' cell phenotype. Cells expressing the high molecular weight CD45RA were thought to be naive, unprimed T-cells, although they were responsive to mitogens and also appeared to be alloreactive.[261] Although this distinction no longer seems so absolute ($CD45RO^+$ cells can re-express CD45RA[262]), the original finding that antigen-specific responsiveness is largely confined to the $CD45RO^+$ phenotype holds true. Therefore, I sought to establish how cell populations, selectively depleted of $CD45RO^+$ or $CD45RA^+$ cells, responded to sacc and other stimuli. Sacc, SK and TT all elicited considerably greater responses from $CD45RO^+$ cells; by contrast, $CD45RA^+$ cells responded rather better than $CD45RO^+$ to PWM. It is difficult to say how much of the apparent $CD45RA^+$ response to sacc was due to contaminating $CD45RO^+$ cells, since these could not be measured flow cytometrically, having been depleted with an antibody identical to the marker antibody. However, a maximum contamination of 5.9% is possible. If only $CD45RO^+$ cells were responding to sacc, and the response were in proportion to the number of cells present, this degree of contamination could account for an uptake of about half of that observed in the '$CD45RA^+$' population. However, applying the same principle to SK and TT, the observed counts for $CD45RA^+$ cells should have been larger, and so sacc did appear to be slightly less selective for $CD45RO^+$ cells in this single experiment. Of course, the difference may well be within the range of experimental error, and does not alter the overall impression that sacc-specific responses bear greater similarity to those to known recall antigens than to polyclonal mitogens.

If the cellular response to sacc is antigen-specific, one would expect only a small proportion of cells in the peripheral blood T-cell population to be capable of recognising it. Experiments were therefore undertaken with the aim of measuring this precursor frequency. Unfortunately, although values for the precursor frequency could readily be derived, in three out of four attempts using cells from the same donor, the results could not be shown to conform to single-hit kinetics. This could have been because of inherent variability of the data (see esp. Fig. 22b, p. 92). However, on inspection of the data plots, there was a suspicion that the fraction of responder wells at low cell inputs may have been disproportionately low, with data points for cell inputs <1000/well always lying on the same side of the 95% confidence interval for the data as a whole.[a] This could mean that the technique as described is insufficiently sensitive in detecting positive wells with a low number of clonal precursors. This situation should give a multi-hit curve (concave upwards towards the cell input axis, on the plots used here);[172] this did not seem to be the case visually and the means for testing this statistically were not available. In any case, re-analysis of the most complete data set (Fig. 22d), either by omitting the data point from the lowest cell input, or by taking 2 SD above background as the threshold for a positive response, gave results compatible with single-hit kinetics. As expected, the first method resulted in the least alteration to the original estimate of the precursor frequency (an increase by a factor of 1.07, *c.f.* 1.2 for the second method). The first attempt at measuring SK-specific precursor frequency gave a result compatible with single-hit kinetics. (It is worth noting that, paradoxical as it may seem, the more complete the data set, the more power the analysis has to reject single-hit kinetics, since any observed departure

a: others have also observed this (Dr. X.N. Wang, Newcastle University, personal communication)

from ideal behaviour is less likely to have arisen by chance.) The frequency estimates for sacc and SK were very similar. Furthermore, they are in keeping with other published estimates of frequencies of responder cells to recall antigens.[219–222] My estimate of the sacc-specific cell frequency of 1/1,150 converts to one of 1/600 if only $CD4^+$ cells are considered, and higher still if only $CD45RO^+$ cells participate in the response. This suggests significant levels of sensitisation to a common dietary antigen within the circulating T-cell population in at least some healthy subjects. Although these experiments were not controlled by estimating the precursor frequency to a mitogen, the ease with which cells could be cloned after stimulation with PHA (see below) suggested that this was virtually 100%.

Finally, using unselected T-cell clones grown with PHA and IL-2, and lines and clones grown by multiple rounds of stimulation with sacc and IL-2, it was possible to show that only cells grown in the presence of sacc responded to it, and that other recall antigens were ineffective at inducing proliferation in these cells. However, it must be stated that the cloning efficiency of sacc-specific cells was very low on limiting dilution — the results presented here represent the only success among many failed attempts. Furthermore, for unknown reasons, the proliferation results shown in Fig. 26 could not be reproduced after further rounds of stimulation. Lines and clones could not be maintained indefinitely because of the regular requirement for donor-specific APCs, and so further studies were not possible.

That sacc-stimulated cells have enhanced cytotoxicity for NK-sensitive, and to a lesser extent, NK-resistant target cell lines, suggests that the activity of sacc may not be confined to effects directly attributable to antigen-specific T-cells. The studies on which this experiment was based (see p. 27) were designed to examine the ability

of *Candida*-derived preparations to act as 'biological response modifiers', with possible tumoricidal activity, by stimulating MHC-independent cell killing. However, in the results presented here, cytotoxic activity was possessed to a greater extent by cells stimulated with PPD, and it seems entirely possible that this *in vitro* phenomenon is merely secondary to enhanced NK/LAK cell activity consequent upon IL-2 release by proliferating antigen-specific T-cells. Unfortunately, no parallel data on thymidine uptake are available for this experiment, and so it is not possible to assess the extent to which the cytotoxic response was dependent on the degree of proliferation in culture. It might be possible to imagine circumstances in which this stimulation of cytotoxicity might be biologically significant, for example, in inflamed bowel, but this is highly speculative. Nevertheless, it is interesting to note that enhanced NK activity on exposure to gliadin has been observed in PBMC from patients with coeliac disease.[263]

I did not find convincing evidence for an enhanced cellular response to sacc among Crohn's disease patients. This is in contrast with the results of Young *et al.*,[264] who found a mean SI of 15.6 in 12 Crohn's disease patients, compared with 3.3 in 15 healthy subjects after only three days in culture. The antigenic preparation used by this group was live whole yeast, and nystatin was included in the culture medium. Otherwise, there is no obvious explanation for the discrepancy between these findings.

Is sacc antigen identical to mannan?

Heelan *et al.*[265] subjected a preparation identical to sacc to sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and found a single band, under reducing and non-reducing conditions, which migrated with an apparent

molecular weight of 200 kD and stained weakly with Coomassie blue and strongly with periodic acid Schiff's reagent — evidence for a high carbohydrate content. Following transfer to nitrocellulose membrane, immunoblotting with anti-sacc antibody-positive sera (previously assessed by ELISA) from patients with Crohn's disease, or hyperimmune rabbit serum, demonstrated binding of antibody to this high molecular weight band. In addition, binding of rabbit anti-sacc serum was diminished in a dose-dependent fashion by pre-treatment of the nitrocellulose strips with varying concentrations of periodate, suggesting that carbohydrate epitopes were essential for antibody recognition.

On the basis of these findings, and previous work on *C. albicans* (see p. 27), a high molecular weight fraction of sacc was prepared by collecting material which eluted in the void volume of a Sepharose CL6B column. This preparation, termed 'gp 200', which had similar electrophoretic mobility to the band demonstrated by Heelan *et al.*, stimulated thymidine uptake in PBMC from three different donors who were selected on the basis of previous responsiveness to sacc. However, it is not possible to conclude with certainty that gp 200 is the only component of sacc with this capacity, since lower molecular weight components were not available for testing. Although gp 200 was not assessed independently for antibody-binding, it seems reasonable to suppose that it would have shared this characteristic with sacc.

Recently, Young *et al.*[266] carried out a detailed analysis of the antibody-binding component of sacc. They separated sacc by size-exclusion chromatography on Sepharose 4B. A fraction of apparent high molecular weight (>200 kD) by SDS-PAGE — which stained with PAS and, when used as antigenic substrate in an ELISA, bound antibody from known positive IgG anti-sacc serum — was then

subjected to further gel filtration on a Superose 6 column, which had been calibrated with dextrans for more accurate molecular weight determination. A broad peak of antibody-binding activity was eluted within a molecular weight range of 360–620 kD, and a mean of MW 460 ± 20 kD was confirmed by size-exclusion chromatography/multi-angle laser light scattering (SEC/MALLS) on a HPLC system. This product, termed Sc 500, was found to comprise 90% carbohydrate and 10% protein by weight (2,790 and 440 residues per molecule, respectively). The carbohydrate was mannose with a trace of *N*-acetylglucosamine, with methylation analysis revealing 1→2, 1→3 and 1→6 linkages; serine and threonine each represented over 20% of the amino acid residues of the protein, with asparagine (or aspartic acid) at 9%, and smaller proportions of other amino acids. Treatment of Sc 500 with periodate or alkaline borohydride reduced its ability to inhibit antibody-binding in a competitive ELISA with native Sc 500 as primary antigen (IgG- and IgA-positive human sera and a murine IgM monoclonal antibody, *prepared by immunisation with gp 200*,[267] were the sources of antibody), whereas pronase, trypsin and peptide-*N*-glysosidase F were ineffective. In addition, treatment with a broad-spectrum α-mannosidase, with activity against 1→2, 1→3 and 1→6 linkages, was effective at reducing the capacity of Sc 500 to inhibit binding of the monoclonal IgM. Thus Sc 500 is a high molecular weight species which is similar to gp 200 in three respects: *i)* appearance on SDS-PAGE, *ii)* antigenic cross-reactivity, *iii)* dependency of antibody-binding on carbohydrate structure. Furthermore, it has the composition expected of cell wall mannan of yeast.

I have made parallel assessments of the effects of protein digestion of sacc on its ability to compete with native sacc for antibody-binding and on its capacity to

stimulate proliferation of PBMC. An attempt has also been made to confirm the effectiveness of the enzymatic degradation by assaying the protein content of sacc before and after digestion, with the use of appropriate controls to allow for the presence of inactive enzyme protein. Following protein digestion, antibody-binding capacity was maintained (in keeping with the results of Young *et al.*, above), but lymphocyte proliferation was abrogated, and at the higher dose of enzyme, completely abolished, and this was commensurate with a reduction in measurable protein content. Therefore, it could be proposed that the B-cell epitopes in sacc are protein-independent, whereas the T-cell epitopes are (at least) protein-dependent. However, the first of these conclusions is not entirely safe: because sacc is a mixture, it cannot be assumed in this experiment that the B- and T-cell epitopes are on the same molecule; it may be the case, therefore, that B-cell epitopes are present on, for example, mannan, that these *are* dependent on intactness of the protein component, but that the latter is neither measurable by dye-binding, nor accessible to enzymatic digestion (because of the high degree of glycosylation), while the T-cell epitopes which are being detected by the *in vitro* lymphocyte response are present independently on another protein, which is both measurable and degradable. This question could only be resolved by testing the effect of protein digestion on the lymphocyte-stimulating ability of a more pure mannan preparation, such as Sc 500; to date, this has not been done. Finally, no assessment has been made of the carbohydrate-dependency of the lymphoproliferative response; it is possible to envisage, for example, that sugar side chains may participate in binding of the relevant peptide(s) to MHC class II molecules and/or subsequent T-cell recognition. Evidence for the participation of sugar residues in MHC-peptide-T-cell interaction has

been found in a murine model.[268]

In an attempt to further investigate the possible identity of sacc antigenic activity with that of mannan, I compared sacc with two different 'mannan' preparations — a commercial preparation of *S. cerevisiae* mannan precipitated with CTAB, and a crude mannan which I prepared according to a published method. Both methods should result in a relatively undegraded product. When corrections were made for relative protein and carbohydrate content, the behaviour of sacc and crude mannan were very similar in both lymphocyte proliferation assays and ELISA inhibition, respectively. The commercial mannan failed to stimulate PBMC or inhibit antibody-binding in the sacc-specific ELISA. These last results were unexpected for two reasons: firstly, CTAB mannan from *C. albicans* does stimulate PBMC *in vitro*, secondly, this preparation of *S. cerevisiae* mannan was used as antigenic substrate in an ELISA by Lindberg *et al.*,[5] who found that it behaved very similarly to antigenic material from whole yeast cells. However, in this study, the protein content of the commercial mannan was very low (< 0.4% by weight) — although it was said to have been purified by CTAB precipitation, there is no available information on how it was *extracted* from whole cells; since it is possible that it may have been degraded in the process, this could explain the low protein content and the absence of a T-cell response. With respect to antibody-binding, the use of two substrates in independent ELISAs is not equivalent to assessing the ability of one to inhibit antibody-binding to the other; thus it is conceivable that there are strain-specific differences, or, once again, differences due to degradation during the production process, between sacc and the commercial mannan, resulting in poor cross-reactivity. (Since nothing is known about the T-cell epitopes involved in mannan-specific cellular responses, it is not

possible to say whether these exhibit strain variation.)

Although it remains the most likely candidate, it cannot be assumed that cell wall mannan is the only relevant antigen in sacc, with respect to both humoral and cellular immune responses. Other yeast antigens are certainly capable of being recognised immunologically. For example, acid phosphatase of *C. albicans* is a periplasmic enzyme of high molecular weight (124–136 kD), and also a mannoprotein (with mannose:protein ratio 7:1). Sera from humans with candidiasis have been found to contain precipitating antibody to the purified enzyme; antigenic activity, but not enzyme activity, was heat stable, suggesting that the binding site was on the glycosidic moiety.[269] Acidic carboxyl proteinase is another exocellular manno-enzyme (MW 45 kD) to which infected humans raise antibodies.[270] It is also possible that these glycoproteins share common antibody-binding motifs with mural mannan. If antibody-binding by sacc is wholly mannan-dependent, then it should be completely inhibitable by a highly purified mannan, such as Sc 500; as with the putative T-cell response to Sc 500, this experiment remains to be performed.

The question arises as to whether the demonstration of anti-sacc antibodies could represent a secondary phenomenon due to a response initiated by some other, cross-reacting, antigenic stimulus. Cross-reactivity between yeasts has been recognised: for example, between A and B serotypes of *C. albicans*,[131,271,272] and between *C. albicans* type B and *S. cerevisiae*.[273] Furthermore, Nnalue *et al.*[274] reported that one of eight monoclonal antibodies raised against *Salmonella thompson*, and which was specific for the O-antigen polysaccharide of serogroup C_1 *Salmonellae*, cross-reacted with 142 of 148 *Candida* species tested, as well as 4 of 64 strains of *E. coli*. However, the fact remains that yeast species and strains *are* capable of being

distinguished serologically due to the presence of non-promiscuous determinants. Furthermore, the question of cross-reactivity was addressed by McKenzie *et al.*,[2,3] who found no difference in *Candida*-specific antibodies between normal and Crohn's disease subjects in the presence of highly significant differences for *S. cerevisiae*. Moreover, although Barnes *et al.*[4] found an increased prevalence of *E. coli* antibodies in Crohn's disease, this was equally the case in ulcerative colitis, whereas prevalence of anti-sacc was raised only in Crohn's disease. In a recent publication, Sendid *et al.*[275] have proposed that a mannotetraose structure with an $\alpha 1 \rightarrow 3$ and two $\alpha 1 \rightarrow 2$ linkages is the dominant B-cell epitope responsible for the enhanced antibody response associated with Crohn's disease. If this is the case, whether or not Crohn's sera show increased binding activity for antigen derived from a particular yeast species or strain will depend on its relative expression of this motif. Thus, although it is possible that the apparent specificity of antibody for *S. cerevisiae* may have arisen because of an unrecognised cross-reaction, there is currently no evidence to support this conclusion.

CONCLUSION. The experimental work presented in this thesis confirms the observation of enhanced systemic humoral immunity to *S. cerevisiae* in Crohn's disease, to the yeast, *S. cerevisiae*. This was detected using an unfractionated aqueous extract (sacc), to which *in vitro* cellular responses are readily detectable in normal individuals. Different experimental approaches suggest that the cellular response is antigen-specific, and that the participating cells are $CD4^+$, $CD45RO^+$ T-cells. Evidence to date supports the hypothesis that the dominant B-cell epitopes in sacc reside on the yeast cell wall mannan, but the nature of the T-cell epitopes responsible for the sacc-

induced lymphoproliferative response is less clear, and awaits re-examination with substrates whose composition is better-defined.

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APPENDICES

Appendix I: buffers and solutions

CARBONATE-BICARBONATE BUFFER, pH 9.6: *ELISA-plate coating buffer*

0.05 M Na_2CO_3	5.3 g/l	16 vols. (add to increase pH)
0.05 M $NaHCO_3$	4.2 g/l	34 vols. (add to decrease pH)

Mix in approximate proportions shown and titrate to pH 9.6

PHOSPHATE-CITRATE BUFFER, pH 5: *OPD buffer*

0.1 M citric acid	21.01 g/l	1 vol. (add to decrease pH)
0.1 M $Na_2HPO_4 . 2H_2O$	17.8 g/l	1 vol. (add to increase pH)

Mix in approximate proportions shown and titrate to pH 5

0.05% PBS-TWEEN: *ELISA washing buffer*

Add 5 ml *Tween 20 (Sigma)* to 10 l PBS.

AET: *preparation of SRBC*

Dissolve 4.03 g AET in ~905 ml water, titrate to pH 9 with 4 M NaOH and make up to 100 ml

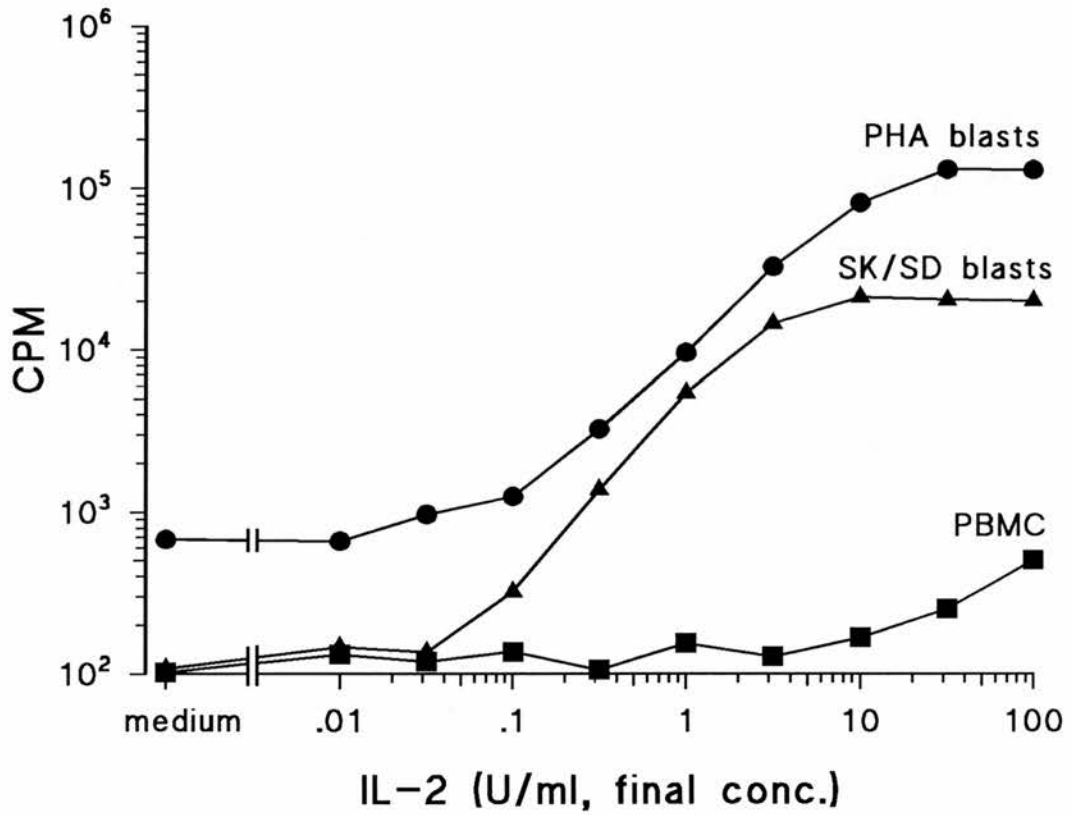
ERYTHROCYTE LYSING BUFFER

10 mM $KHCO_3$	0.2 g
150 mM NH_4Cl	1.6 g
0.14 mM EDTA	0.1 g

Make up to 200 ml in distilled water, adjust to pH 7.2, sterile filter and freeze aliquots.

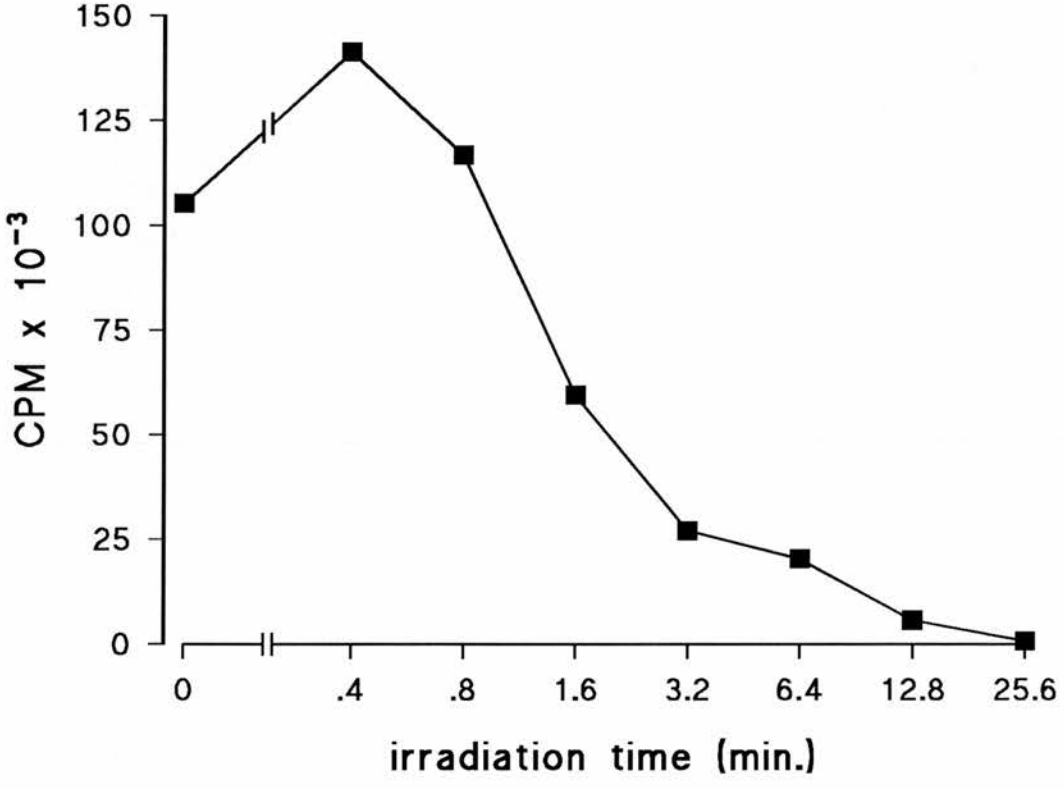
Appendix II: titration of IL-2

The figure below shows the extent of proliferation supported by the preparation of IL-2 alluded to in MATERIALS AND METHODS. PBMC were cultured in the presence of optimal concentrations of PHA or SK for 3 or 7 days, respectively, after which they were washed and incubated in fresh medium with the concentrations of IL-2 shown. The response of fresh PBMC is also shown. Thymidine uptake was measured after 4 days.



Appendix III: effectiveness of cell irradiation

The figure below shows the upake of tritiated thymidine by PHA-stimulated PBMC on the third day after various periods of irradiation. In experimental work, cells were invariably irradiated for 15–20 minutes.



Immunology 1994 **81** 247–252

In vitro human lymphocyte proliferative responses to a glycoprotein of the yeast *Saccharomyces cerevisiae*

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SUMMARY

Following reports of enhanced humoral immunity to *Saccharomyces cerevisiae* in patients with Crohn's disease, and identification of an immunodominant, high molecular weight glycoprotein (gp200), we have investigated the cellular immune response to this yeast in normal individuals. Following exposure to a crude saline extract (Sacc), peripheral blood mononuclear cells (PBMC) from these subjects demonstrated dose-dependent increases in tritiated thymidine incorporation, the time-course of which resembled that of the response to the known recall antigens PPD and TT. This was accompanied by increased cytotoxicity of the cultured cells for natural killer (NK)-sensitive and NK-resistant target cell lines. Furthermore, using a purified, high molecular weight, glycoprotein fraction of Sacc in culture, a dose-dependent lymphoproliferative response was again observed. Stimulation indices (SI) for thymidine incorporation by umbilical cord blood lymphocytes exposed to Sacc were low compared with those of normal adults. These results provide evidence for possible antigen-specific, cellular, immune sensitization of normal individuals to a ubiquitous dietary component.

INTRODUCTION

Recently, Main *et al.* using the enzyme-linked immunosorbent assay (ELISA) technique with a crude saline extract of *Saccharomyces cerevisiae* as solid phase antigen, reported the presence of specific IgG and IgA antibodies in the serum of patients with Crohn's disease, and which distinguished this group from normal controls and patients with ulcerative colitis.[1] In the case of antibodies of the IgG isotype, this observation held true for 11 of 12 *S. cerevisiae* strains examined, and there was no serological cross-reactivity with two strains of *Candida albicans*.[2] Results from this laboratory confirmed and extended these findings, suggesting that IgA anti-Sacc antibody, though less sensitive, was a more specific marker for Crohn's disease;[3] furthermore, there was no correlation between the presence of anti-Sacc antibodies and those reacting with *Escherichia coli* or other dietary antigens. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by periodate oxidation and immunoblotting with antibody-positive sera suggest that the relevant antigen is a high molecular weight (~200,000 MW), heat-stable, soluble glycoprotein (gp200).[4]

The demonstration of specific humoral immunity to this putatively non-pathogenic organism, quantitatively different in subjects with a specific pathology as compared with normal individuals, raises the possibility that concomitant cellular immunity to the same antigen is also present. The fact that *in vitro* immune responses to the pathogenic fungus *C. albicans* have been extensively studied[5–11] has provided a model for the study of this related organism and we have previously reported preliminary findings of increased *in vitro* DNA synthesis by peripheral blood mononuclear cells (PBMC) in response to a crude saline extract of the yeast (Sacc), which suggest that this is the case even in normal subjects.[12,13]

The aim of the present work has been to confirm and extend these observations by making a qualitative comparison between lymphocyte proliferative responses to Sacc and those to recognized recall antigens and mitogens, thereby establishing optimal culture conditions with which to explore the phenomenon of cell-mediated cytotoxicity induced by Sacc. The lymphoproliferative response to the previously identified high molecular fraction of Sacc has also been examined.

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MATERIALS AND METHODS

Preparation of cells

PBMC were obtained from normal subjects by the method of Böyum.[14] Briefly, aliquots of whole blood were collected from donors by venepuncture and diluted with sterile, heparinized (15 U/ml final concentration) physiological saline (ratio of blood : saline = 1 : 1 to 2 : 1). This mixture was then separated on a Lymphoprep (Nycomed, Oslo, Norway) density gradient by centrifugation at 2000 rpm (~830 *g*) for 20 min at 20°. The buffy layer was removed by pipette and the cells washed once in an excess of wash fluid [RPMI-1640/25 mM HEPES (Flow Laboratories, Irvine, U.K.) plus heparin at 15 U/ml)] followed by centrifugation at 1400 rpm (~400 *g*) for 15 min at 20°. After discarding the supernatant, the pellets were resuspended in a small volume of complete culture medium [RPMI/HEPES, each

100 ml supplemented with the following: 3 ml 7·5% $NaHCO_3$ solution (Gibco, Paisley, U.K.); 10 ml heat-inactivated (56° for 30 min), sterile-filtered (0·2 μm), human AB serum; 2 ml L-glutamine 200 mM (Gibco) and 2 ml of a solution containing penicillin 5000 U/ml plus streptomycin 5000 μg/ml (Gibco)] prior to counting. Cells obtained by this method were virtually 100% viable by trypan blue exclusion.

Heparinized umbilical cord blood was first depleted of erythrocytes by preincubating each 10 ml aliquot with 2 ml 1% methylcellulose (Sigma, St Louis) in phosphate-buffered saline (PBS) at 37° for 15 min with mixing, then diluting with an equal volume of physiological saline and allowing to settle for a further 30 min prior to density gradient centrifugation of the supernatant suspension with Lymphoprep.

Antigens and mitogens

Sacc. A saline extract of *S. cerevisiae* was prepared as previously described.[4] Briefly, 100 g of a commercially available dried bakers' yeast (Sainsbury's) was suspended in 500 ml sterile physiological saline at 4° and washed twice, resuspending to the original volume after each centrifugation. Following the second wash, the suspension was heated in a water-bath at 100° for 1 hr. After cooling, the suspension was centrifuged, and the supernatant sterile filtered to 0·2 μm. Total protein concentration was measured by the Coomassie brilliant blue colorimetric method (Bio-Rad, Richmond, CA), and the preparation was stored in aliquots at −20° until further use.

A purified high molecular weight fraction was prepared by subjecting an aqueous extract (as above, but prepared in water rather than saline) to an approximately sixfold volume reduction by ultrafiltration using a TCF 2-micro-thin channel ultrafiltration system (Amicon, Lexington, MA) with membranes having a 50,000 MW cut-off; the ultrafiltrate was then lyophilized and that product was then applied to a Sepharose CL6B column (Pharmacia, Uppsala, Sweden) equilibrated in sterile water, and the fraction eluting in the void volume was collected and lyophilized. This fraction showed a single band of apparent MW = 200,000 after SDS–PAGE and staining with Coomassie brilliant blue and periodic acid Schiff's reagent (PAS).

Purified protein derivative of Mycobacterium tuberculosis *(PPD).* A pharmaceutical preparation containing 100,000 U/ml PPD (Evans Medical, Horsham, U.K.) was obtained. This had a total protein concentration of 750 μg/ml and was stored until further use at 4°, according to the manufacturer's recommendations.

Tetanus toxoid (TT). Unadsorbed tetanus vaccine, 60 ml (Evans Medical) was dialysed extensively against distilled water, sterile filtered (0·2 μm), and lyophilized; it was then redissolved to 10 ml in water, centrifuged, refiltered and stored at −20° until further use.

Pokeweed mitogen (PWM). A stock solution was prepared by dissolving 10 mg lyophilized mitogen (Sigma) in 5 ml sterile water. Aliquots were stored at −20° until further use.

Interleukin-2 (IL-2). A stock solution of purified lymphoblastoid IL-2 (Biotest, Dreieich, Germany) containing 100,000 U/ml was stored at 4°.

Proliferation assays

For dose–response and time–course experiments, PBMC were adjusted to $2/3 \times 10^6$ lymphocytes/ml in culture medium and plated on to sterile 96-well, U-bottomed culture plates (Flow) at 10^5 cells in 150 μl/well. Dilutions of antigens/mitogens were prepared in culture medium at four times their intended final concentrations in culture and added to the wells in 50 μl volumes; the same volume of culture medium alone was added to control wells. Plates were then incubated for variable periods at 37° in a humid atmosphere containing 5% CO_2.

On the day of harvesting, the cells were pulsed with [methyl-^{3}H]thymidine (1 mCi/ml) (Amersham International, Amersham, U.K.) by adding 1 μl to each well and incubating for a further 6 hr. The cells were then harvested on to glass fibre filters using a semi-automatic cell harvester (Flow). The filters were allowed to dry overnight prior to β-counting of the individual discs in "OptiScint 'Hi Safe'" liquid scintillant (LKB, Surrey, U.K.).

Medians were calculated for each group of replicate wells. Results are expressed either as absolute c.p.m. or as the stimulation index (SI: count for stimulated culture/count for corresponding unstimulated control culture).

Cytotoxicity assay

Effector cells. PBMC were cultured in bulk at 5×10^5 lymphocytes/ml in 50-ml flasks and under the same incubation conditions as described above for the proliferative assay. Four cultures were established for each donor, as follows: unstimulated cells; cells plus Sacc; cells plus PPD; cells plus IL-2. After 7 days, the cells were washed in RPMI supplemented with 10% fetal calf serum (FCS), resuspended in a small volume of the medium, and a viable count (trypan blue exclusion) performed. Finally, the cells were adjusted to 5×10^5/ml.

Target cells. Two cell lines were used: mel-1, a natural killer (NK)-resistant, malignant melanoma-derived cell; and Molt4, an NK-sensitive, T-cell leukaemia-derived cell. These were harvested from culture, washed twice in RPMI/FCS and the centrifuged pellets were resuspended in the presence of 10 μl of a solution of ^{51}Cr-sodium chromate (10 mCi/ml) (Flow). After incubation for 1 hr at 37°, the cells were washed twice in RPMI/FCS and adjusted to 5×10^4/ml after performing a viability count.

The assay was performed by adding 150 μl of each effector cell suspension to the same volume of each target cell suspension in 11-mm diameter test-tubes in triplicate, to give an effector to target cell ratio of 10:1. After gentle centrifugation at 1,000 rpm (~208 *g*) for 5 min, followed by incubation for 4 hr at 37°, 150 μl of supernatant was removed from each tube. Respective pairs of pellets and supernatants were then counted in a γ-counter. Spontaneous release was calculated from tubes containing target cells plus 150 μl medium only, and maximum release from tubes containing target cells plus 150 μl 2% Triton 100 (BDH) to lyse the cells. A mean background count was subtracted from each experimental count, and the per cent cytotoxicity for each pair of tubes calculated as follows:

$$\% \text{ cytotoxicity} = \frac{R_{test} - R_{spont}}{R_{max} - R_{spont}} \times 100$$

where: R_{test} = fractional release in test sample; R_{spont} = mean spontaneous fractional release; R_{max} = mean maximum fractional release. The mean of each triplicate was taken as the final result.

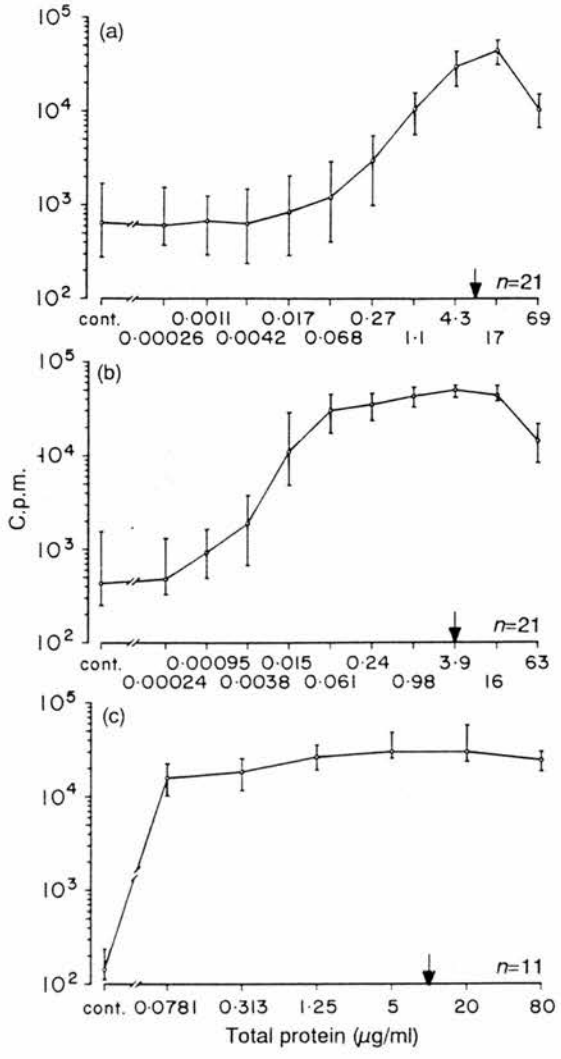

Figure 1. Dose–response relationship for: (a) Sacc; (b) PPD; (c) PWM. In (a) and (b), eight replicate wells were used for each subject at each dose, and six replicates in (c); the final response for each group of replicates being the median of the individual counts. These medians were then used to estimate the population median and the 95% confidence interval (Wilcoxon) at that dose. Figures on the horizontal axes refer to final concentrations in culture; cont. = control, medium only added to culture. Arrows indicate concentrations used for the subsequent time–course experiment.

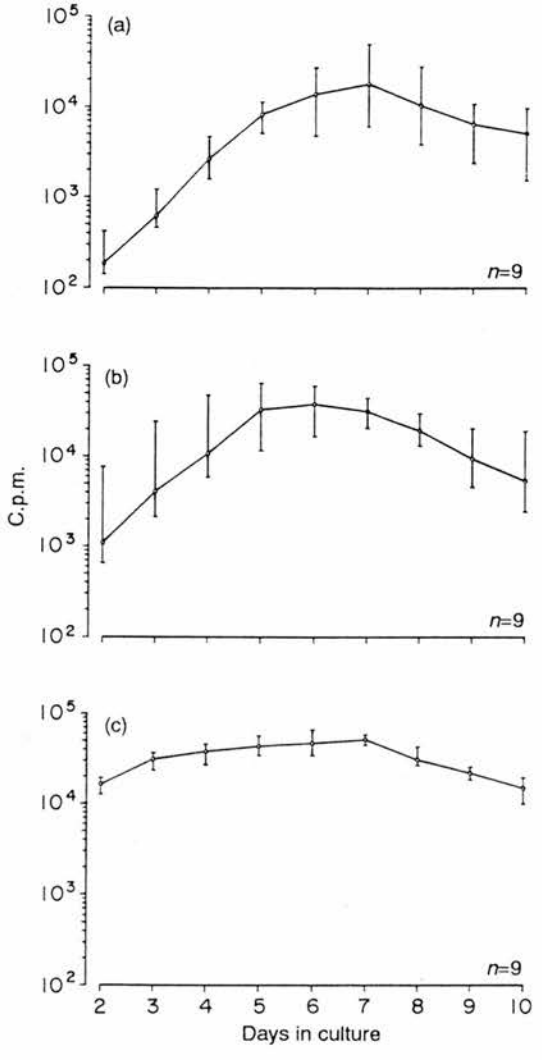

Figure 2. Time–course of response to: (a) Sacc (8·6 μg/ml); (b) PPD (3·9 μg/ml); (c) PWM (10 μg/ml). Each experiment was performed on six replicate wells and the analysis was as for the data in Fig. 1.

Statistical analysis

Data were compared using the appropriate non-parametric tests in MINITAB (MINITAB, State College, PA), for personal computers.

RESULTS

Dose–response and time–course

After 7 days in culture, the proliferation, as measured by tritiated thymidine incorporation over 6 hr, of PBMC from normal donors in response to a range of fourfold dilutions of Sacc, PPD and PWM is shown in Fig. 1, where the median and 95% confidence interval for the sample is plotted at each concentration. A major feature is that the demonstration of an optimal response to Sacc appears to be more critically depen-

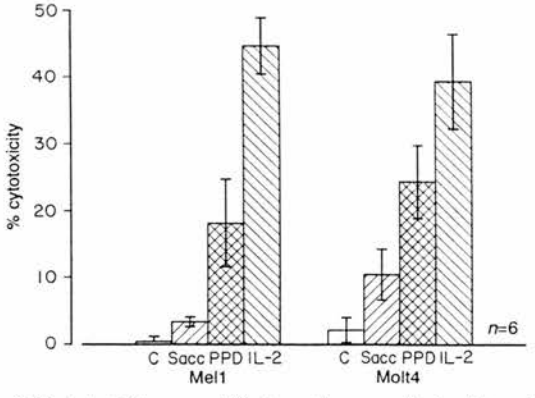

Figure 3. Cytotoxicity assay. Effector cells were obtained by culture of lymphocytes from six normal donors: C = control (unstimulated) cells. Concentrations of Sacc and PPD used for preculture of effector cells were as for Fig. 2; IL-2 was used at 50 U/ml. Bars show mean ± SEM % cytotoxicity by effector cells, incubated in a ratio of 10:1 with target cells.

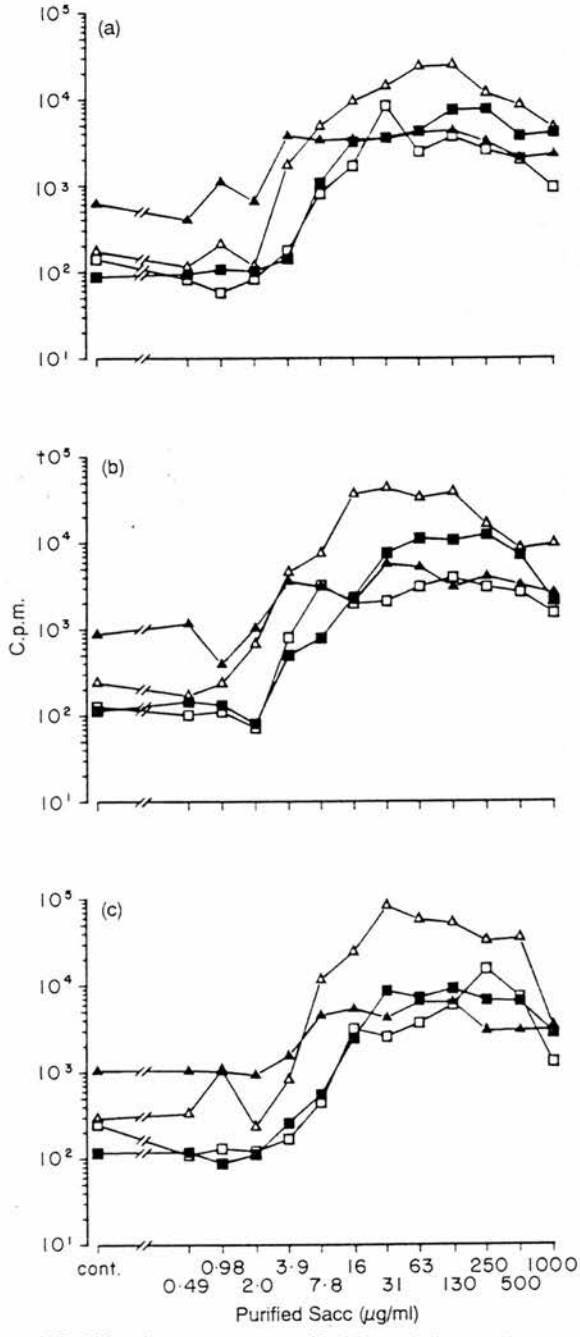

Figure 4. Proliferative response to purified Sacc. Cells were harvested on 3 consecutive days: (a) day 6; (b) day 7; (c) day 8. Each data point is the median of five replicates. Separate dose–response curves are shown for each of the four subjects.

dent on dose than is the case for PPD and PWM, which both produce a shallow plateau of responses over a greater than 200-fold concentration range.

Data on the time-course of the proliferative responses (Fig. 2) were obtained by measuring tritiated thymidine incorporation daily in the presence of that concentration of Sacc, PPD and PWM which had produced the optimal response at 7 days (these are indicated by arrows against the horizontal axes in Fig. 1). Whereas PWM elicited very early proliferation which was maintained throughout the course of the experiment, peak

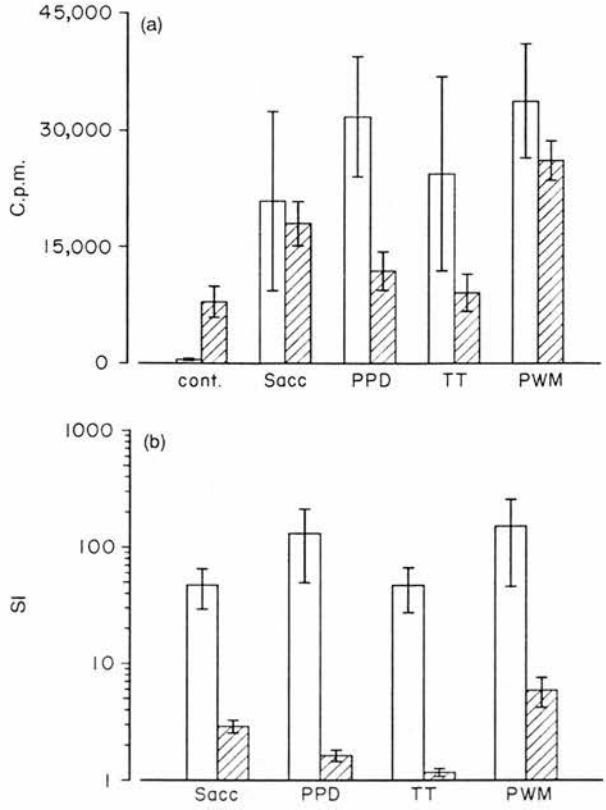

Figure 5. Comparison of proliferative responses of PBMC (□, $n=4$) and CBMC (▨, $n=9$), expressed as: (a) absolute c.p.m.; (b) SI. Bars show mean ± SEM derived from five replicates.

responses to Sacc and PPD were delayed at 7 days and 5–6 days respectively.

The dose dependency and time-course of the response to TT (not shown) closely resembled that of PPD.

Cytotoxicity

The per cent cytotoxicity obtained against the mel-1 and Molt4 cell lines with an effector to target cell ratio of 10:1 is shown in Fig. 3. Similar results were obtained for ratios of 20:1 and 5:1 (data not shown). All effector cell samples demonstrated cytotoxicity significantly greater than that of the unstimulated control at $P<0{\cdot}05$ (Wilcoxon signed rank test). Antigen-stimulated cells elicited greater killing against the NK-sensitive line (Molt4) compared with the NK-resistant line (mel-1).

Proliferative responses to purified Sacc

PBMC were obtained from four donors who had previously demonstrated significant proliferative responses to Sacc and tested for their ability to proliferate in response to doubling dilutions of a high molecular weight fraction purified by gel filtration. Figure 4 shows that the responses were comparable regardless of whether cells were harvested after 6, 7 or 8 days in culture. Furthermore, it can be seen that the amount of thymidine incorporation is less critically dependent on dose than was the case for the unfractionated Sacc preparation, and in this respect the response more closely resembles those of PPD and TT.

Proliferative response of cord blood lymphocytes

Cord blood mononuclear cells (CBMC) were incubated with concentrations of Sacc, PPD, TT and PWM which had been shown to be optimal for adult PBMC. After 7 days in culture, the incorporation of tritiated thymidine was compared with that of similar cultures of normal adult PBMC (Fig. 5). Although the responses, in absolute c.p.m. of stimulated CBMC cultures were in each case not significantly different from those of PBMC (Fig. 5a), unstimulated CBMC cultures uniformly demonstrated a high background uptake [$P < 0{\cdot}01$ (Mann–Whitney) cf. PBMC] with consequently reduced SI for stimulated cultures [$P < 0{\cdot}01$ (Mann–Whitney) cf. PBMC] (Fig. 5b).

DISCUSSION

Saccharomyces cerevisiae is not recognized as a pathogen except in rare, anecdotal cases. However, exposure to this organism in the form of bakers'/brewers' yeast is virtually universal and the demonstration of humoral immunity to the yeast extract Sacc, specifically in relation to Crohn's disease,[1-4] raises the possibility of sensitization via the gut-associated lymphoid tissue (GALT), a site at which activated T lymphocytes may be involved in induction or maintainance of gastrointestinal disease.[15]

Using incorporation of tritiated thymidine as a marker of DNA synthesis in PBMC, it has been possible to establish dose–response relationships for Sacc, PPD and PWM after 7 days in culture (Fig. 1). Although the profile of the Sacc response appears to differ markedly from those of PPD and PWM, in so far as optimal thymidine uptake is more critically dependent on a narrow range of concentrations, this may have been an artefact due to the nature of the preparation used: that is, above a concentration of total Sacc protein of 17 μg/ml it may not have been possible to increase the proportion of the saline extract in the culture medium without either introducing significant amounts of inhibitory factors or excessively diluting essential components of the medium. Indeed, this anomaly was not observed with the purified, lyophilized, high molecular fraction (see below).

Having thus established appropriate optimal concentrations, the time-course of each response was examined (Fig. 2). The kinetics of the responses to Sacc and PPD are similar and contrast with that to PWM in that the maximum responses are delayed, reaching well-defined peaks on day 7 (Sacc) and day 6 (PPD), compared with the early plateau of thymidine uptake seen with PWM. It could be argued that this observation supports the suggestion that Sacc, like PPD, is mediating lymphocyte activation in an antigen-dependent manner rather than acting non-specifically as a mitogen. This conclusion might be criticized on the grounds that a maximal response at about 7 days has already been preselected on the basis of the doses chosen and that the earlier response to PWM merely reflects less stringent dose requirements rather than a fundamental difference in the kinetics of the response *per se*. However, when tetanus toxoid was used as recall antigen, it also resulted in maximum uptake at 5–7 days, and this remained true over a greater than 200-fold dose range (results not shown). The kinetics of the response to Sacc are in broad agreement with those reported elsewhere for antigenic preparations of *C. albicans*.[5,6]

In previous studies in mouse and man, *C. albicans*-stimulated cells have been shown to possess *in vitro* cytotoxic activity against a variety of allogeneic targets and this has been attributed to induction of NK cell activity.[7-9] Sacc also appears to have this property: the cytotoxicity of Sacc-stimulated cells for both NK-sensitive and NK-resistant cell lines was statistically greater than that demonstrated by negative control cells (Fig. 3). The levels of cytotoxic activity induced by Sacc were less than those obtained with the control antigen PPD, and this may reflect differences in the precursor frequency or phenotype of lymphocyte subsets which each activates. In the studies on *C. albicans* cited, cytotoxicity was also greater, and although the different target cell lines, and in some cases, higher effector: target cell ratios used in those experiments make direct comparison with the current data difficult, Sacc does appear to be a less potent inducer of cytotoxicity than *C. albicans*.

The ability of a purified high molecular weight glycoprotein fraction of Sacc to elicit a lymphoproliferative response was examined, and, as is shown in Fig. 4, this retained similar efficacy to the crude preparation. This fraction had the electrophoretic and staining characteristics of the previously identified gp200,[4] properties also shared by a heat-stable mannoprotein fraction of *C. albicans* which was recognized by anti-*Candida* rabbit serum and has been demonstrated to induce lymphoproliferation and cytotoxicity *in vitro*.[9]

Umbilical cord blood contains T cells which are said to be phenotypically and functionally immature.[16] In some studies, *in vitro* CBMC responsiveness to a stimulating substance, for example, mannan of *C. albicans*,[17] has been taken to imply mitogenicity. However, weak CBMC responses to antigenic stimuli are detectable, and, in the case of food antigens, have been proposed as predictors of future allergy.[18] In addition, limiting dilution analysis has demonstrated a precursor frequency of PPD-specific cells in cord blood only 10- to 100-fold less than adult peripheral blood, and no difference in the frequencies of cells from these populations which respond to the 65,000 MW mycobacterial heat-shock protein.[19] Because cell preparations may differ in their spontaneous rate of thymidine uptake, the SI is often used as a device to normalize data from lymphoproliferation assays; we, and others,[16] have found CBMC to generate particularly high background counts. Although results manipulated in this way should be interpreted with caution, our experiments showed CBMC responses to Sacc, PPD and TT which, expressed as SI, were significantly less than those of PBMC, whereas there was no difference between cell populations when results were expressed in c.p.m. Microscopic visual inspection of the cultures tended to support the conclusion that significant proliferation of CBMC only occurred in response to PWM; in this latter instance, the apparently low SI may have been due to the fact that it was measured after the peak response had been achieved.

In conclusion, in keeping with the apparent ability of *S. cerevisiae* to elicit humoral immune responses in man *in vivo*, soluble preparations of the organism can induce a lymphoproliferative response *in vitro* which is kinetically similar to that due to known recall antigens. This property is retained by a high molecular weight fraction and is accompanied by the ability to induce cytotoxic activity in stimulated cells. Whether the response of PBMC to Sacc is truly antigen specific remains to be established, as the evidence presented here, based on the kinetics of the response and hyporesponsiveness of CBMC, is merely suggestive. However, it has been shown (C. J. Darroch, S. E. Christmas and R. M. R. Barnes, unpublished observations) that

randomly selected T-cell clones, which proliferate in response to PWM and phytohaemmagglutinin, are not stimulated by Sacc, and work is in progress to attempt to isolate clones which are specifically responsive to Sacc.

Although the biochemical nature of the active component(s) of Sacc has not been fully characterized, the method of preparation, possession of electrophoretic and staining characteristics of a high molecular weight glycoprotein, and the known immunological properties of yeasts[10,11] make cell wall mannan the most likely candidate (preliminary analysis of the purified preparation supports this suggestion).

It is not known what relevance the ubiquitous presence of *S. cerevisiae* in food has to these observations. Lymphoproliferative responses to other food antigens are not universally demonstrable in normal individuals;[20] Sacc may be unusual in this respect, and further study is necessary to characterize its role in health and disease.

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